EXHIBIT "A"

CURRICULUM VITAE - PROF. DR. HERJAN J.T. COELINGH BENNINK

Personal details

Name: Coelingh Bennink
Christian names: Herman Jan Tijmen (Herjan)
Date/place of birth: 14-03-1943, Winterswijk, The Netherlands
Nationality: Dutch
Marital status: Widower; remarried
Children: Three daughters born 1968, 1969, 1971 and eight grandchildren
Hobbies: Music (passive), Art, Literature
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Education/Training

1955 - 1960:	High School, Thorbecke Lyceum, Utrecht
1960 - 1966:	Medical School University of Utrecht (doctoral degree)
1967 - 1969 (Feb. 26):	Medical Degree, Erasmus University, Rotterdam
1967 - 1969:	Medical Assistant, Dept. of Cell Biology, Stichting Klinisch Hoger Onderwijs, Erasmus University, Rotterdam
1969 - 1970:	Residency in Internal Medicine, St. Geertruiden Hospital, Deventer
1971:	Residency in Internal Medicine, Department of Immunohaematology, Bloodbank, Leiden
1971 - 1976:	Residency in Obstetrics and Gynaecology, Academic Hospital, Utrecht
June 1, 1976:	Registration as Medical Specialist in Obstetrics and Gynaecology

Additional training

1974:	Fellowship, Centre for Research in Reproductive Biology, University of Michigan, Ann Arbor, USA (Prof. S.J. Behrman)

Languages

English:	Fluent in speaking and writing
German:	Working knowledge
French:	Working knowledge

Medical positions

1976 - 1985:	Head of Department of Reproductive Medicine and Obstetrics, Department of Obstetrics and Gynaecology, Academic Hospital, Utrecht
1985 - 1987:	Private practice, Juliana Hospital, Veenendaal
1987 – 2000:	Several positions at Organon International (see addendum)
1997 - 2005	Professor of Reproductive Medicine, Free University, Brussels (Heads Prof. P. Devroey and Prof. A. van Steirteghem)
Per October 1, 2000:	CEO and President, "Pantarhei Bioscience"

Scientific experience

University:	Gestational diabetes, Prolactin, GnRH, PCOS, Anorexia Nervosa, IVF
PhD thesis:	Gestational Diabetes, University of Utrecht
Papers:	More than 120 publications in peer reviewed journals
Books:	Editor of 5 books on Reproductive Medicine
Referee:	Regular referee for "Human Reproduction", "Maturitas" and the European Journal of Obstetrics, Gynecology and Reproductive Biology

ADDENDUM TO CV OF PROF. HERJAN J.T. COELINGH BENNINK (HCB)

Research and Development activities at Organon International

In March 1987 HCB joined Organon after a University career as Assistant Professor in Obstetrics and Gynaecology, specialized in Reproductive Medicine (RM).
In his first year at Organon he reorganized the internal International Monitoring Organization and introduced Good Clinical Practice (GCP).
From 1988 until early 1997 he reorganized and managed the RM Clinical Development Department consisting of three groups: Contraception, Infertility and Hormone Replacement Therapy (HRT). He has been responsible for the management and scientific content and quality of on average about 25 clinical RM projects running at 250-300 sites all over the world at any given time.
HCB has initiated and supervised the clinical development of new regimens of combined oral contraceptives, progestagen only pills (POP), new progestagens, a progestagen implant, new IUD's, a combined contraceptive vaginal ring, antiprogestagens, hormonal male contraception, new urinary gonadotrophins, recombinant human FSH, a GnRH antagonist, new recombinant gonadotrophins, tissue specific steroids for HRT and more conventional estrogen replacement regimen. During his Directorship of Clinical RM R&D he has completed six phase III projects, comparable to NDA's; two on Contraception (Implanon® and the Desogestrel (DSG) POP Cerazette®), two on Infertility (RecFSH; Puregon®/Follistim® and the GnRH antagonist ganirelix (Antagon®) and two on HRT (Livial® and DSG/E2 sequential). HCB has brought the standards of Organon's clinical RM-R&D at the appropriate scientific level, comparable to the highest university standards, confirmed by a rapidly increasing number of peer-reviewed publications in major RM-journals.
Since early 1997 HCB has been responsible for Organon's complete RM-R&D as Director of the RM-Programme. This included shared responsibility for the pipeline, the strategy, the innovative genomics oriented research in biotechnology, biochemistry, pharmacology, pharmacy, toxicology and drug metabolism, but also for the clinical development, technology, production and registration of new RM compounds.
During the first half of 1997 HCB has prepared a plan to innovate Organon's RM research. New concepts were introduced in all three areas of RM, supervised by a Programme Research Committee under his chairmanship. In 2000 in preclinical and clinical development 16 RM projects were running, supervised and managed by five International Project Managers who reported to HCB, whereas he reported to the Managing Director R&D.
Over the years HCB has build up an extensive network of opinion leading experts. He has managerial experience with complicated organisations, budgeting, financial negotiations, patent issues, licensing, communications with authorities (a.o. FDA and EMEA), teaching, lecturing, writing, refereeing papers for scientific journals and organising and chairing (scientific) meetings.
Per October 1, 2000, HCB has resigned from N.V. Organon and has founded Pantarhei Bioscience B.V. in Zeist, the Netherlands per January 1, 2001.

EXHIBIT "B"

CLIMACTERIC 2008;11(Suppl 1):47–58

Estetrol review: profile and potential clinical applications

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Key words: ESTETROL, E_4, REVIEW

ABSTRACT

In this review paper, the existing information on the human fetal steroid estetrol (E_4) has been summarized. In the past, E_4 was considered as a weak estrogen and interest disappeared. However, recent new research has demonstrated that E_4 is a potent, orally bioavailable, natural human fetal selective estrogen receptor modulator, since it acts in the rat as an estrogen on all tissues investigated except breast tumor tissue, where it has estrogen antagonistic properties in the presence of estradiol. Based on its safety data, its pharmacokinetic properties, its pharmacological profile and the results of first human studies, E_4 may be suitable as a potential drug for human use in applications such as hormone replacement therapy (vaginal atrophy, hot flushes), contraception and osteoporosis. Additional areas worth exploring are the treatment of breast and prostate cancer, hypoactive sexual desire disorder and topical use (wrinkles) in women, autoimmune diseases, migraine, cardiovascular applications and the treatment of selected obstetric disorders.

HISTORY

Estetrol (E_4) was discovered by Diczfalusy and co-workers in 1965[1] and was a topic of preclinical pharmacological research thereafter for a period of about 20 years. Studies during that period showed that this estrogenic steroid molecule has four hydroxyl groups[2]. Isolation and identification of this novel estrogen were achieved by extracting 200 liters of late pregnancy urine[3]. On the basis of physical and chemical characteristics, it was concluded that the compound was identical with 15α-hydroxyestriol (15α-OHE_3) or estra-1,3, 5(10)-triene-3,15α,16α,17β-tetrol[3]. It was further concluded that E_4 is synthesized exclusively by the fetal liver during human pregnancy, reaching the maternal circulation through the placenta. This conclusion was based on previous work, which showed that the liver is the exclusive site of 15α- and 16α-hydroxylation[4–6]. The structural formulae of E_4 and other estrogenic steroids are presented in Figure 1, demonstrating that these estrogenic steroids differ in the number of hydroxyl (OH) groups only.

Several ADME (absorption/distribution/metabolism/excretion) properties of E_4 have been studied in postmenopausal and last-trimester pregnant women using parenteral administration of steroids labeled with radioisotopes[7,8]. Estetrol was minimally, if at all, metabolized and was not reconverted to estriol (E_3) or estradiol (E_2). When injected intravenously to adults, it was rapidly and completely excreted in urine as a Ring D monoglucuronide, but otherwise metabolically unaltered[9–11]. According to these data,

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REVIEW ARTICLE

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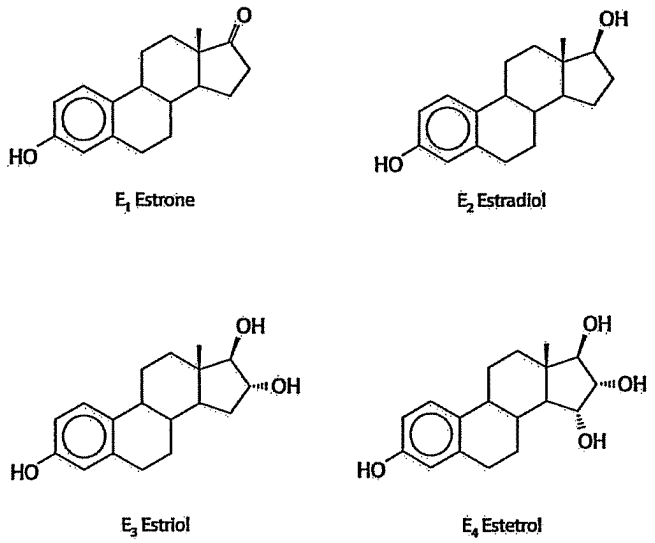

Figure 1 Structural formulae of estrone (E_1), estradiol, (E_2), estriol (E_3) and estetrol (E_4)

E_4 does not appear to enter the enterohepatic circulation.

Estetrol was detected in maternal urine as early as 9 weeks of pregnancy[12,13]. It was found at high levels in maternal plasma during the second trimester of pregnancy, with steadily rising concentrations of unconjugated E_4 to about 1 ng/ml (>3 nmol/l) toward the end of pregnancy[14,15]. Conjugated E_4 levels were seven times higher than unconjugated levels[16]. The levels of unconjugated E_4 in fetal plasma at parturition were about 12–19 times those in maternal plasma[17,18]. Amniotic fluid levels were about one-third of fetal plasma levels and five to six times higher than maternal plasma levels[17,19]. Maternal urinary excretion in late pregnancy varied between 0.5 and 2.3 mg/day[13,20–22].

For follow-up and survey of pregnancy pathology, E_4 levels were not appropriate due to the large intra- and inter-individual variations in plasma levels[23–27].

Competitive receptor binding studies for nuclear binding in human proliferative endometrium[28] and in rat uterine cytosol[29] revealed low estrogen receptor binding affinity for E_4, relative to that of E_2.

The effects of E_4 when compared to those of estrone (E_1), E_2, and E_3 on progesterone receptor levels and growth in the human breast cancer cell line MCF-7 revealed that both E_3 and E_4 behave as E_2 agonists but require substantially higher concentrations to achieve the effects of E_2[30].

Estetrol produced a number of biological changes in the rodent uterus, such as weight increase, progesterone receptor stimulation, enzyme induction, and histological and ultrastructural changes[31]. It also bound to the human endometrial estrogen receptor. The biochemical, histological and ultrastructural responses of the immature rat uterus to E_4 revealed a tendency toward cell differentiation, in contrast to the typical mitotic responses that were observed after E_2 administration[32].

After 20 years of experimental work, E_4 research was virtually abandoned and ended in the mid-1980s. Consensus at that time was that, first, E_4 is a weak estrogen and, second, E_4 cannot be used as a marker of fetal well-being during pregnancy due to the high inter- and intra-individual variations in plasma levels[14,33].

However, it seems unlikely that an estrogenic steroid produced in such significant quantities by the male and female human fetal liver during pregnancy would have no physiological significance. Therefore, in 2001, a project was started at Pantarhei Bioscience to investigate the properties of E_4 with state-of-the-art technologies.

SYNTHESIS AND PHARMACEUTICAL PROPERTIES

A new route of synthesis has been developed for E_4 by Pantarhei, starting with the commercially available E_1[34]. This new route results in accep-

table yields of E_4 of very high purity (>98%) without contamination with E_2. It permits the synthesis of E_4 on a (semi)-industrial scale suitable for GMP (Good Manufacturing Production) for human use[35]. This new method overcomes the disadvantages of previous methods.

Pharmaceutical studies revealed that E_4 is chemically very stable even under non-optimal storage conditions[34]. It has high water solubility and might be slightly hygroscopic. Estetrol has an octanol–water parturition coefficient (Pow) of about 1.5, making it about a 100-fold less lipophylic than E_2 or ethinylestradiol. As a Pow of 2 is considered optimal for passage through the blood–brain barrier, E_4 might be expected to have effects on the central nervous system.

It is concluded that, with this new method, E_4 can be synthesized consistently with high purity and without important contaminations. Estetrol appears to have very favorable properties for the development of a pharmaceutical product.

RECEPTOR BINDING AND TARGET INTERACTION

Estetrol has a moderate affinity for human estrogen α receptor (ERα) and estrogen β receptor (ERβ), with K_i values of 4.9 ± 0.567 nmol/l and 19 ± 1 nmol/l, respectively, demonstrating a four- to five-fold preference for the ERα (lower K_i value)[36].

Estetrol has high selectivity for the estrogen receptors. Binding at the glucocorticoid, progesterone and testosterone receptors was only 11–15% at a concentration of 10 μmol/l and further profiling of E_4 in a set of 124 receptors and enzymes demonstrated inactivity towards 123 molecular targets. The single target showing interaction with E_4 was the adrenergic $\alpha_{1\beta}$ receptor (weak binding)[36].

It is concluded that genomic clinical effects of E_4 will most likely occur through the estrogen receptors. The high selectivity of E_4 suggests a low risk of unexpected side-effects.

LIVER CELL METABOLISM AND PROTEIN BINDING

The rate of metabolism of E_4 was studied in rat and human hepatocytes and was found to be slow in both *in vitro* systems[36], complying with the observed slow elimination and long half-life *in vivo*[37,38].

The metabolites found after incubation of E_4 with rat and human hepatocytes were completely different[36]. Metabolites produced by rat hepatocytes were not found with human hepatocytes and *vice versa*. In rat hepatocytes, phase I metabolism is most important. This may result in active metabolites in the rat, whereas inactivation by glucuronidation and sulfation, i.e. phase II metabolism, are the pathways observed in human hepatocytes[36]. This confirms that, in the human, E_4 is an end-stage product of metabolism and has no active metabolites.

Estetrol at a high concentration of 10 μmol/l did not inhibit the major cytochrome P450 enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP 3A4[36]. Estradiol and ethinylestradiol significantly inhibited CYP2C19. Ethinylestradiol had a strong inhibitory effect on CYP3A4, whereas E_2 stimulated this enzyme significantly and E_4 had some stimulatory effect[36]. These results suggest that E_4 may exhibit less interference with concomitantly administered drugs (drug–drug interaction) compared to ethinylestradiol and E_2.

The ERα-dependent effect of the steroids E_2, E_3, E_4 and ethinylestradiol on sex hormone binding globulin (SHBG) production was investigated using the HepG2 and Hep89 cell lines[39]. Estetrol did not stimulate the production of SHBG in both cell lines, suggesting that E_4 may not influence the plasma levels of SHBG. The estrogens E_2, E_3 and ethinylestradiol all show a dose-dependent ERα-mediated increase in the production of SHBG. This increase in SHBG production is most prominent for E_2, while addition of E_3 and ethinylestradiol resulted in a lower and comparable increase in SHBG. Binding of E_4 to SHBG was also studied *in vitro*[39]. There was no detectable binding of E_4 to the estrogenic and androgenic human SHBG steroid-binding sites (Figure 2). By contrast, testosterone and E_2 were bound with high affinity, whereas the synthetic estrogen ethinylestradiol binds to SHBG with low affinity[39]. These data indicate that SHBG has no influence on the plasma distribution of E_4 or its availability to target tissues, contrary to other natural steroid ligands such as E_2 and testosterone and several synthetic progestins that all bind to SHBG.

ADME AND ORAL BIOAVAILABILITY

Earlier ADME studies were performed using parenteral administration of radiolabeled steroids[7,8,33], but oral administration of E_4 was never considered in the past. Since oral treatment was considered crucial for the potential use of E_4

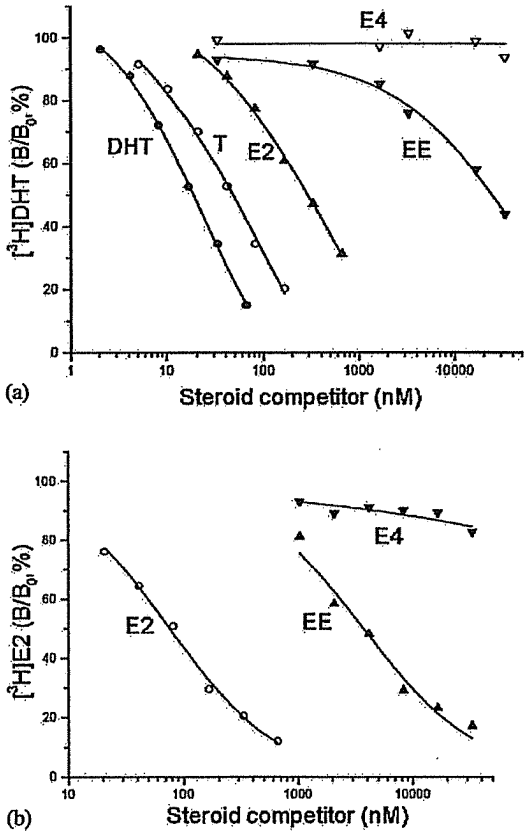

Figure 2 Competitive displacement of [^{3}H]DHT (a) and [^{3}H]E_2 (b) from the human sex hormone binding globulin (SHBG) steroid binding site. The unlabelled steroid ligands used as competitors were as follows: DHT, 5 -dihydrotestosterone; T, testosterone; E_2, estradiol; EE, ethinylestradiol; E_4, estetrol

as a drug for human use, its oral bioavailability and pharmacokinetics were studied as the very first stage of the new E_4 research in the rat[37]. When E_4 appeared to have favorable oral kinetics in the rat[37], at a later stage pharmacokinetic studies were performed in the human[38].

In the bioavailability study in the rat, E_4 was administered as a single dose of 0.05, 0.5 or 5.0 mg/kg orally or subcutaneously to female rats[37]. Plasma was analyzed using an LC-MS/MS method. Oral bioavailability of E_4, relative to that of subcutaneous dosing, was 70% and above at the 0.05 and 0.5 mg/kg doses, based on the AUC. Subcutaneous dosing provided significantly higher E_4 levels at the 1-h time point only, and was comparable to oral dosing after 0.5, 2, 4 and 8 h (Figure 3). So, rather surprisingly, E_4 was found to have a high oral bioavailability in the rat[37]. Also the elimination half-life observed after 2–3 h is relatively long, since the rat liver is known to be very efficient in metabolizing steroids.

These findings had at least two implications. First, the oral bioavailability enabled once-daily oral treatment with E_4 in further studies in the rat, a species considered to be relevant and predictive for the human. Second, the pharmacokinetic data obtained in the rat suggested that oral treatment with E_4 might be possible also in the human.

A pharmacokinetic study with E_4 was performed in healthy early postmenopausal women. Four single doses of 0.1, 1, 10 and 100 mg E_4 were administered to six women each[38]. It was shown that E_4 is absorbed orally very effectively with high dose–response relationship and low inter-subject variability (Figure 4). The elimination half-life of E_4 was found to be 28 h, suggesting slow metabolism of E_4 and suitability for once-a-day oral administration. The pharmacokinetic pattern suggested enterohepatic recirculation[38]. Pharmacokinetic simulations with special emphasis on AUCs were performed using these human pharmacokinetic data and E_4 levels during pregnancy[14,15] (see also History). Based on these calculations, the term fetus synthesizes about 3 mg E_4 per day, whereas the term fetal exposure to E_4 is more than 50 mg/day when compared to oral administration of E_4 to postmenopausal women.

In summary, the old and new ADME data obtained with E_4 support its potential as an oral once-a-day drug for human use.

BONE

The bone-sparing effect of oral E_4 compared to that of ethinylestradiol was studied in ovariectomized rats[37]. The use of the ovariectomized rat model for the preclinical evaluation of drugs intended for prevention and treatment of osteoporosis is recommended by the US Food and Drug Administration[40]. Once-daily oral treatment with E_4 by a dose of 0.1, 0.5, or 2.5 mg/kg/day, or by 0.1 mg/kg/day of ethinylestradiol as positive control, was given for 4 weeks. The following measurements were performed:

(1) Serum osteocalcin,
(2) Bone mineral density, bone mineral content and bone mineral area of lumbar vertebrae L3–L6,
(3) Peripheral quantitative computed tomography of the left tibiae, and
(4) The biomechanical properties (strength) of the distal femora.

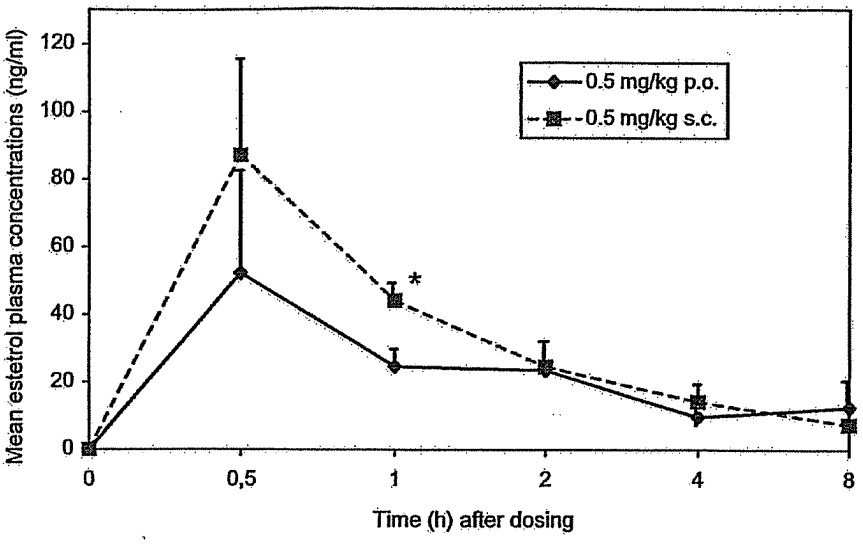

Figure 3 Mean (±standard deviation) plasma concentrations of estetrol after oral (p.o.) or subcutaneous (s.c.) administration of a single dose of 0.5 mg/kg estetrol to female rats ($n=3$). *Significantly different from vehicle: $p<0.01$

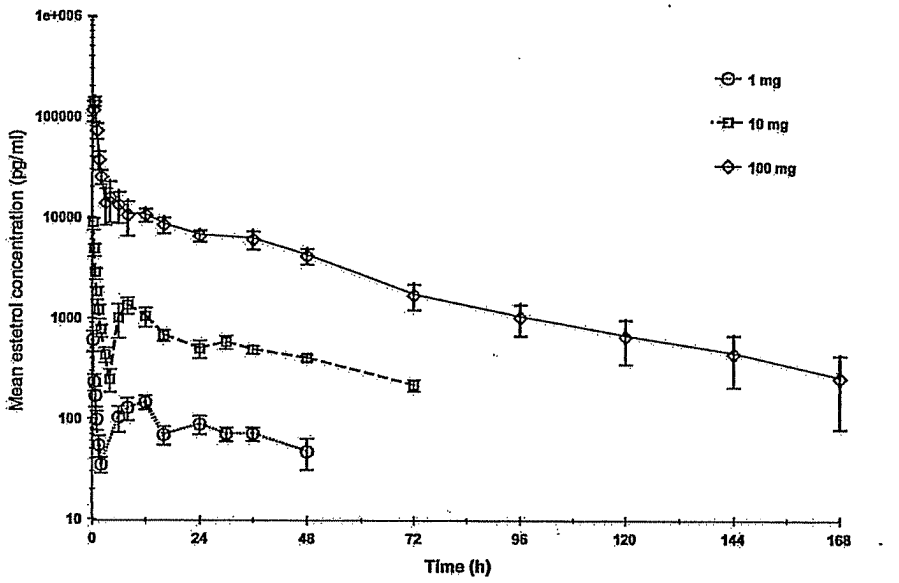

Figure 4 Mean estetrol plasma levels (±standard deviation) after a single oral dose of 1, 10 and 100 mg estetrol in postmenopausal women

Estetrol dose-dependently and significantly inhibited the ovariectomy-related increase in osteocalcin levels, increased bone mineral density and content and increased bone strength (Figure 5), all attenuated by ovariectomy. In this rat model, the relative potency of the highest dose of E_4 of 2.5 mg/kg/day was comparable to the 0.1 mg/kg/day ethinylestradiol dose, used as positive control. It was concluded that oral administration of E_4 conveys dose-dependent bone-sparing effects of high-quality bone in estrogen-depleted ovariectomized rats[37].

Based on its bone-sparing effects and its oral bioavailability in this highly predictive and

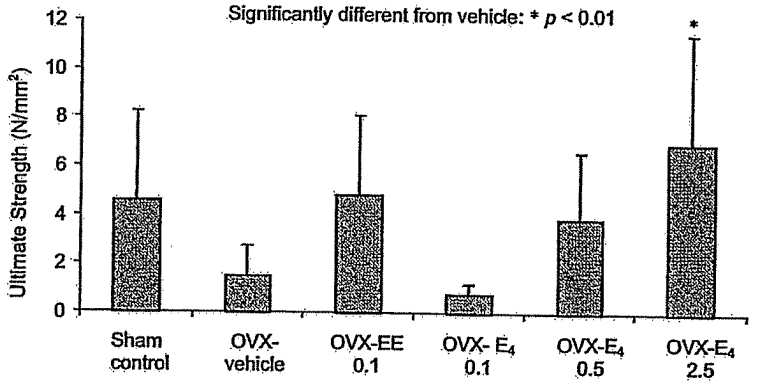

Figure 5 Mean (±standard deviation) ultimate strength (N/mm^2) at the distal femora after 4 weeks of once-daily treatment with estetrol (E_4) (0.1, 0.5 or 2.5 mg/kg/day) or ethinylestradiol (EE) (0.1 mg/kg/day) compared to vehicle in ovariectomized (OVX) rats and sham-operated controls

well-validated animal model, E_4 is a potential drug for the prevention of osteoporosis in postmenopausal women. It seems worthwhile also to investigate the potential efficacy of E_4 for the treatment of osteoporosis and osteoporotic fractures.

HOT FLUSH

The efficacy of E_4 in alleviating hot flushes was studied in an experimental rat model considered representative for menopausal vasomotor symptoms[41]. In this model, the thermal responses in the tail skin of morphine-dependent ovariectomized rats are recorded after administration of naloxone. Six groups of rats were treated orally for 8 days as follows: vehicle (negative) control; E_4: 0.1, 0.3, 1.0 and 3.0 mg/kg/day; and as active (positive) control ethinylestradiol: 0.3 mg/kg/day. On day 8, tail skin temperature was recorded at baseline and for 60 min at 5-min intervals following naloxone administration. In control animals, tail skin temperature increased sharply by about 4.5°C after naloxone treatment and reverted to baseline by 60 min. Estetrol suppressed the tail skin temperature increase in a dose-dependent fashion (Figure 6). The highest dose of E_4 tested (3 mg/kg/day) was equipotent to a 10-fold lower dose of ethinylestradiol. Both fully suppressed tail skin temperature changes[41].

It is concluded that E_4 is effective in preventing temperature rises in an experimental animal model considered representative for studying the effect of drugs on the menopausal hot flush (vasomotor symptoms). In this model, the potency of E_4 was 10-fold lower compared to ethinylestradiol.

These results suggest that E_4 may be effective for the treatment of hot flushes and other vasomotor symptoms in peri- and postmenopausal women.

VAGINA, UTERUS AND ENDOMETRIUM

The effect of E_4 on vaginal cornification and uterine weight was studied in ovariectomized rats[42]. Six groups of rats were treated orally once daily for 7 days as follows: vehicle (negative) control; E_4: 0.1, 0.3, 1.0 and 3.0 mg/kg/day; and ethinylestradiol 0.05 mg/kg/day as active (positive) control. Vaginal lavages were obtained daily and, on day 7, uterine wet weight was determined. Vaginal cornification was observed by day 5 in all rats at all E_4 doses and in the animals receiving ethinylestradiol, but not in the control rats (Figure 7). The onset of cornification with E_4 was dose-dependent. After 7 days treatment, the two highest E_4 doses (1.0 and 3.0 mg) induced statistically significantly higher uterine wet weight (myometrium) compared to vehicle[42].

In the pharmacological study with E_4 to investigate oral bioavailability and prevention of bone loss[37], the uterus of the ovariectomized rats was excised after 4 weeks of treatment. Wet uterine weight (myometrium) was estimated and histological investigation of the endometrium was performed. Four weeks of E_4 treatment induced dose-dependent increases in uterine weight of ovariectomized rats. In this model, the potency of ethinylestradiol in increasing uterine weight was 5–25 times higher than that of E_4.

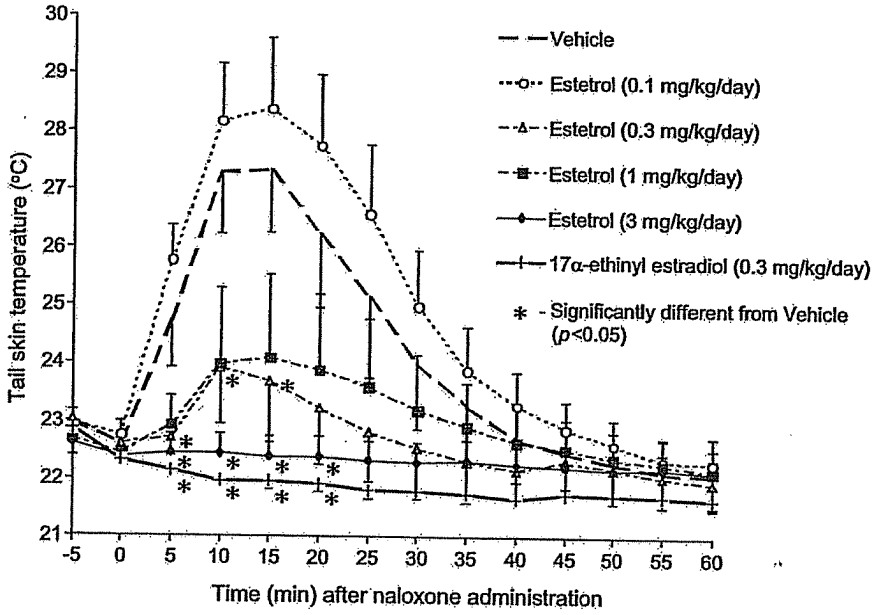

Figure 6 The effects of estetrol (E_4) and 17α-ethinylestradiol (EE) on the naloxone-induced hot flush response in female ovariectomized rats

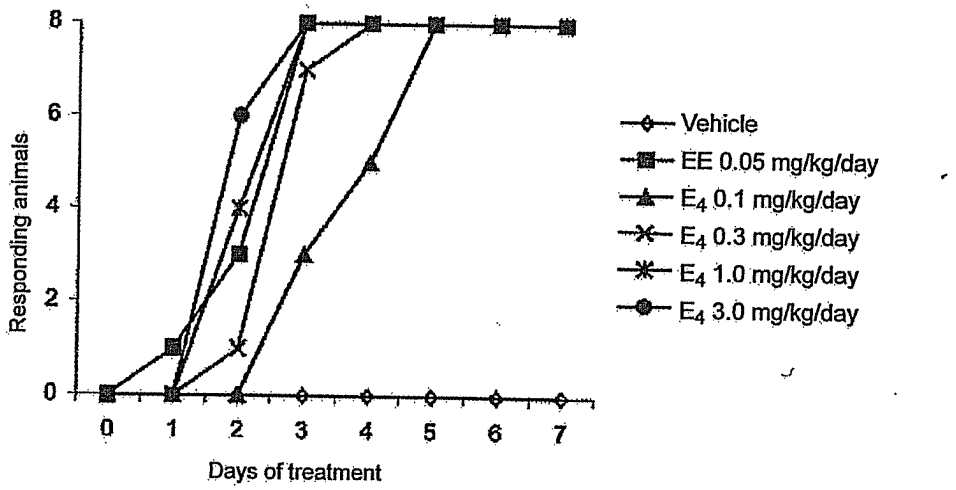

Figure 7 Number of animals with vaginal cornification over a 7-day treatment period with orally administered estetrol (E_4) (0.1, 0.3, 1.0 or 3.0 mg/kg/day), ethinylestradiol (EE) (0.05 mg/kg/day), or vehicle

Estetrol appeared to have a dose-dependent proliferative estrogenic effect on the rat endometrium after 4 weeks' treatment[37]. Estetrol was found to be less potent than ethinylestradiol, since the order of increasing potency per mg/kg/day was estimated as follows: 0.1 mg E_4 <0.5 mg E_4 <0.1 mg ethinylestradiol <2.5 mg E_4.

In summary, estrogenic activity of E_4 was demonstrated in three tissues in ovariectomized rats: vaginal epithelium, myometrium and endometrium. The potency of E_4 was approximately 20-fold lower compared to ethinylestradiol.

It is concluded that E_4 may be suitable for the treatment of urogenital atrophy and the accompanying clinical complaints such as vaginal dryness and dyspareunia in estrogen-deficient women. Since E_4 has a proliferative effect on the endometrium, in women with a uterus, measures

to protect against endometrial hyperplasia and cancer should be taken.

BREAST

Rats treated with DMBA (7,12 dimethylbenz(a)-anthracene) develop estrogen-responsive breast tumors. Two prevention studies and one intervention study were performed in this animal model with E_4[43]. In the prevention studies, the effect was investigated of oral doses of E_4 over a dose range of 0.5–3.0 mg/kg. The intervention study used oral doses of 1, 3 and 10 mg/kg E_4. The antiestrogen tamoxifen was used as reference compound in all three studies; ovariectomy and ethinylestradiol, at doses pharmacologically equipotent to E_4, acted as control treatments in one prevention study and in the intervention study.

When DMBA-induced rats were co-treated with E_4 for 8 weeks, this resulted in a dose-dependent reduction in the number and size of tumors, an effect that appeared equally effective as tamoxifen treatment or ovariectomy and was not seen with ethinylestradiol. When E_4 was administered to rats in which tumors had already developed, a significant decrease in the number and size of tumors was observed after 4 weeks. This decrease was dose-dependent, comparable to tamoxifen-treated animals, and, at high dose levels, E_4 was as effective as ovariectomy (Figure 8)[43].

It was concluded that E_4, dose-dependently, prevents the growth of chemically induced mammary tumors in female rats and has the potential to reduce the number and size of such mammary tumors when already present.

Based on these results, one may hypothesize that E_4 could be a natural antagonist of the abundantly available estrogens during human pregnancy. These findings provide the basis for the potential clinical development of E_4 as a natural selective estrogen receptor modulator (natural SERM) and antagonist for the treatment of breast cancer. The estrogen antagonistic effect on breast tumor tissue would be a major advantage compared to presently used estrogen-containing drugs.

OVULATION INHIBITION

The effectiveness of E_4 as an ovulation inhibitor was studied in regularly cycling female rats and compared to ethinylestradiol[44,45]. The animals were treated orally twice daily for 4 consecutive days, starting on the day of estrus, with E_4 (0.03, 0.1, 0.3, 1.0 or 3.0 mg/kg), or ethinylestradiol (0.0003, 0.001, 0.003, 0.01 or 0.03 mg/kg) or vehicle control. The primary endpoint was the number of ovulated oocytes in the genital tract. Estetrol at the twice-daily dose of 0.3 mg/kg and above inhibited ovulation. This effect was statistically significant ($p < 0.05$). The comparator, ethinylestradiol, significantly inhibited ovulation ($p < 0.05$) at the highest dose (0.03 mg/kg) administered twice daily. The ED_{50} for the ethinylestradiol and the E_4 dose–response curves show that ethinylestradiol is 18 times more potent than E_4 (Figure 9)[44]. In summary, twice-daily administration of E_4 effectively inhibits ovulation in cycling rats. The effect is dose-dependent. The relative potency of E_4 is about 18 times less compared to that of ethinylestradiol.

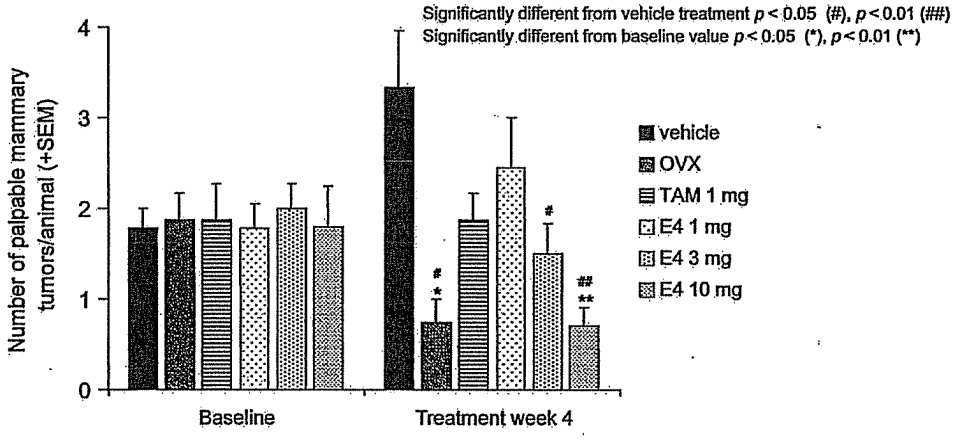

Figure 8 Intervention study. Mammary tumor count per animal (+ SEM) at baseline and 4 weeks after ovariectomy (OVX) or daily oral treatment with vehicle, tamoxifen (TAM) or estetrol (E4)

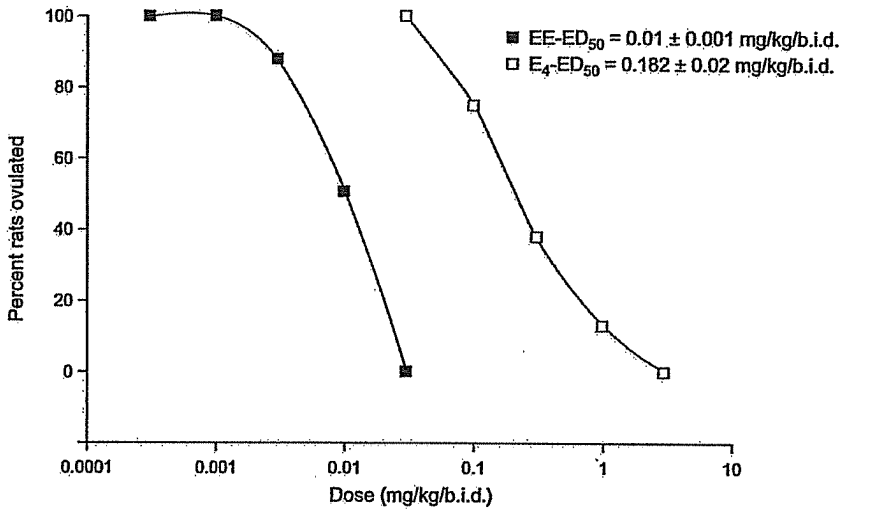

Figure 9 Estimation of ED_{50} for ovulation inhibition in 4-day cycling rats treated twice daily with the indicated oral doses of ethinylestradiol (EE) or estetrol (E_4)

In the human pharmacokinetic study performed with single doses of 0.1, 1, 10 and 100 mg E_4, the effect on plasma levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) was also studied. LH levels were suppressed dose-dependently. A profound and sustained inhibition of FSH levels, lasting over 7 days, was observed in the 100 mg dose group (FSH was not measured in the other dose groups). It was concluded that E_4 has a profound central inhibitory and dose-dependent effect on gonadotropins, expected to contribute to the contraceptive effect of E_4.

In summary, based on these and other results reported in this review, E_4 might be a potential candidate to replace ethinylestradiol in combined oral contraceptives.

CONCLUSIONS

Estetrol is a steroid synthesized exclusively by the human fetal liver during pregnancy. After its discovery in 1965 by Egon Diczfalusy and co-workers at the Karolinska Institute in Stockholm, Sweden, basic research was performed on E_4 until about 1984. At that time there was consensus that E_4 is a weak estrogen and interest in this steroid discontinued. The judgement of low potency of E_4 was primarily based on low ER binding affinity, ranging from 0.3% (rat)[29] to 6.25% (human)[28], and 2–3% potency compared to E_2 in a series of *in vitro* and *in vivo* experiments[30–32]. Efforts to use E_4 as a marker of fetal well-being during pregnancy failed due to the high inter- and intra-individual variations in plasma levels[23–27].

In 2001, Pantarhei Bioscience started its research activities on E_4. The motivation was the simple consideration that it seems unlikely that nature would endow the human fetus with the ability to produce this steroid in such significant amounts without a sound reason.

The new research has revealed that, first of all, E_4 seems a safe compound with selective binding to both estrogen receptors[36] and some preference for the ERα above the ERβ. No toxicity was observed at high dose levels in pharmacological studies in the rat, with maximum doses of 10 mg/kg/day for 4 weeks[43] and in single-dose studies in the human, with a maximum dose of 100 mg[38]. Based on its chemical[34], pharmaceutical[34] and metabolic properties[14,33,37,38], it seems possible to develop E_4 as a drug for human use. Especially its high and dose-related oral bioavailability in the rat[37] and the human[38], the absence of binding to SHBG[39] and the long elimination half-life of 28 h in the human[38] may enable its use as an oral once-a-day drug.

In well-validated and predictive rat models, E_4 behaves as an estrogen agonist in all tissues investigated, i.e. bone[37], vagina[42], myometrium[42], endometrium[42] and brain (hot flush[41] and ovulation inhibition[44,45]), except for breast tumor tissue where this steroid acts as an estrogen antagonist in the presence of E_2[43]. Interaction with liver function in *in vitro* models demonstrates, first, slow metabolism[36], explaining the long half-life; second, absence of cytochrome P450 inhibition[36],

which may implicate less drug–drug interaction; and, third, no stimulation of SHBG synthesis[39], which suggests a potentially lower risk of venous thromboembolism when used as a drug in the human, a serious side-effect of all known estrogens and synthetic SERMs.

Based on its pharmacological profile, E_4 can be classified as a natural human fetal SERM. Contrary to the conclusion in the past, E_4 seems to be a potent steroid. In the pharmacological studies, E_4 was 10–20 times less potent compared to ethinylestradiol, the most potent estrogen available. Single doses of E_4 strongly suppressed LH and FSH in postmenopausal women. The difference between the past and the present conclusions can be explained by (lack of) knowledge of the metabolic properties of E_4. Past studies were all *in vitro* or short *in vivo* experiments and adequate ADME studies demonstrating the favorable pharmacokinetics of E_4 were performed only recently[37,38].

Estetrol may be useful for a series of potential clinical applications including the prevention and treatment of osteoporosis and hormone replacement therapy in women, especially for the treatment of vaginal atrophy and hot flushes. Estetrol seems also suitable as the estrogenic component in oral contraceptives. The effect of E_4 on breast cancer seems worthwhile of being investigated in view of the results in the rat DMBA model. All these possible applications should be explored in clinical proof-of-concept studies. Furthermore, it seems interesting to study the effect of E_4 in autoimmune diseases that are related to thymocyte-1 (Th-1) function such as multiple sclerosis, rheumatoid arthritis and Sjögren's syndrome, since Th-1-related diseases are known to improve considerably during pregnancy when E_4 is present. Studies with E_4 in animal models for multiple sclerosis (EAE model) and rheumatoid arthritis (CIA model) have shown a significant and dose-dependent favorable effect (data not shown). Additional areas worth exploring are the treatment of prostate cancer, hypoactive sexual desire disorder and topical use (wrinkles) in women, migraine, cardiovascular applications and the treatment of selected obstetric disorders.

Although there are sound reasons to test the use of E_4 in all these conditions, it seems unlikely that E_4 will be efficacious in all these disorders and diseases. However, the present data on E_4 allow the conclusion that the pharmacological profile of E_4 is not a weak estrogen but a potent steroidal SERM with potential applications in the human.

The question remains about the physiological role of E_4 during human pregnancy since this has not been studied and is unknown. The facts are that E_4 is a steroid synthesized exclusively by the human fetal liver during pregnancy[1–6]. Data on file show that E_4 is not synthesized by pregnant rats and mares. Estetrol is already present in urine of pregnant women at 9 weeks of gestation[12,13]. Estetrol plasma levels increase exponentially during pregnancy and the term fetus synthesizes a high amount of E_4 up to 3 mg/day. Unanswered questions are, for example:

(1) Why is E_4 present during pregnancy?
(2) How is 15α-hydroxylation during pregnancy regulated?
(3) Why is the expression of this enzyme restricted to pregnancy?
(4) How is the fetus protected against the estrogenic activity of E_4?

These and other questions may be elucidated by further estetrol research dedicated to unravel the *raison d'être* of this intriguing natural human fetal steroidal SERM.

Conflict of interest H.C.B. is CEO and shareholder of Pantarhei Bioscience, the company developing estetrol; C.F.H. has financial interest in estetrol.

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EXHIBIT "C"

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HETEROGENEITY OF SATURABLE ESTRADIOL BINDING SITES IN NUCLEI OF HUMAN ENDOMETRIUM. ESTETROL STUDIES

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SUMMARY

Competition of [^{3}H]-estradiol (E_2), estetrol (E_4) and ethynyl estradiol (EE) for binding to estrogen receptors in human endometrial cytosol was studied at various competitor/estradiol (C/E_2) ratios. The fraction of E_2 that remains bound to the receptor in the presence of competitor (f) was measured at different concentrations of the competitor. Straight lines were obtained by plotting $(1-f)/f$ vs C/E_2, a result that would be expected if E_2, E_4 and EE compete for the same set of binding sites in endometrial cytosol. The relative values of association constants of E_2, EE and E_4, estimated from the slopes of the regression lines, were 100:70:1.5. Scatchard analysis of binding of each of these labeled compounds yielded values for relative association constants which were in agreement with those from competition studies. Similar concentrations of specific binding sites were obtained using labeled E_2, E_4, and EE.

These results indicate that previously published data on competition of E_4 and E_2 for nuclear binding in human endometrium, interpreted to reveal heterogeneity in estrogen binding sites in the nucleus, are not due to heterogeneity in cytosolic E_2 receptors.

INTRODUCTION

Results of *in vitro* studies on the competition of estradiol (E_2) and estetrol (1,3,5-10 estratriene, 3,15α, 16α, 17β-tetrol, E_4) for saturable binding in human endometrium have been previously reported [1]. The published report described the observation that incubation of tissue slices with mixtures of E_2 and E_4 in various proportions yielded the same amount of receptor-bound estrogen ($E_2 + E_4$) in the nuclei. The proportion of E_2 and E_4 bound to receptors in the nucleus depended on their relative concentration in the medium. An unexpected result from those studies was that about 35% of the estradiol binding sites in the nucleus was clearly more resistant to competition by E_4 than the other 65%. In contrast, ethynylestradiol tested in similar experiments displaced all nuclear bound E_2, following kinetics characteristic of competition for a single class of binding sites.

It was then suggested that the observed differences in competitiveness of E_4 and E_2 at various E_4/E_2 ratios were due to a heterogeneity in the nuclear binding of E_2. Great interest in such a possibility derives from the observation that the amounts of E_2 needed to obtain maximal binding to receptors in rat uterus *in vivo* are higher than those needed for maximal biological effect [2]. It is possible that disproportion in binding and biological action may correlate with heterogeneity in nuclear estradiol binding sites.

The main purpose of the present study was to examine the competition of E_2 and E_4 for binding to receptors in endometrial cytosol, in order to determine whether the heterogeneity in binding sites, evident after interaction of the estrogen–receptor complex with nuclear acceptors, might already exist at the cytoplasmic receptor level.

MATERIALS AND METHODS

Tissue. Human endometrium specimens, obtained after dilatation and curettage or from excised uteri, were immediately transported to the laboratory, cleaned under cold saline, and frozen at −80°C for the preparation of cytosol. All endometria studied were proliferative and histologically normal.

Labeled and unlabeled steroids. Labeled steroids, [6,7-^{3}H] E_2 (S.A.: 48 Ci/mmol), [6,7-^{3}H] EE (S.A.: 40.9 Ci/mmol), and [2,4-^{3}H] E_4 (S.A.: 52 Ci/mmol), were purchased from New England Nuclear Corp. Their radiochemical purity was ascertained by mixing aliquots with authentic standards and measuring specific activities before and after t.l.c. or crystallization.

Crystalline E_4 was purchased from the Lamar Research Group, E_2 was supplied by Steraloids, EE and diethylstilbestrol (DES) by Sigma Chemical Corp. High pressure liquid chromatography of 50 μg of E_4 carried out on a microporasil column (Waters Associates) using chloroform, indicated that this compound was free (<0.1%) of estrone or estradiol.

Preparation of cytosol. The frozen endometrium was pulverized with a Thermavac apparatus, and

homogenized at 4°C in an all-glass tissue grinder in 4 volumes of 10 mM Tris, 1.5 mM EDTA, 0.5 mM dithiothreitol buffer (TED), adjusted to pH 7.4 at 25°C. The homogenate was centrifuged at 100,000 *g* for 45 min. The supernatant was separated and treated with dextran-coated charcoal (5 mg/ml cytosol) for 15 min at 0–4°C, in order to reduce the concentration of endogenous estrogens. After centrifugation at 2000 *g* for 10 min, the cytosol was diluted with TED buffer to obtain a protein concentration of about 5–10 mg/ml. Protein levels were measured by the method of Lowry [3].

Competition of E_2, E_4, and EE for binding in cytosol. Solutions of [^{3}H]-E_2 in TED buffer (1.92×10^6 d.p.m./ml; 18.4 pmol/ml) were used in the competition experiments. Unlabeled competitors (E_4 or EE) were added to obtain the ratios indicated in Table 1. Cytosol (0.2 ml) and TED buffer solution (0.2 ml), containing either [^{3}H]-E_2 or [^{3}H]-E_2 plus competitor, were incubated for 3 h at 23°C. Time studies have indicated that maximum labeling is achieved under these conditions. At the end of this period, 0.6 ml of the dextran-coated charcoal suspension (2.5 mg/ml Norit A, 0.25 mg/ml dextran T-70 in TED buffer) was added, and the mixture was occasionally shaken for 15 min at 0–4°C. The charcoal was separated by centrifugation at 2000 *g* for 10 min. An aliquot of the supernatant (0.5 ml) was transferred to a counting vial and 10 ml of toluene-based scintillation fluid was added. The mixture was shaken in a Vortex mixer to extract the steroid into the organic phase.

Each incubation was carried out in parallel with another in which unlabeled E_2 was present at a 100-fold higher concentration than [^{3}H]-E_2. The amount of radioactivity bound, corresponding to non-specific binding, was subtracted from the bound radioactivity in the tube to which only [^{3}H]-E_2 was added. This difference was considered to correspond to saturable (specific) binding of E_2 to cytoplasmic receptors.

Each assay was conducted in duplicate.

Binding of [^{3}H]-E_4 (S.A.: 115,000 d.p.m./pmol) or [^{3}H]-EE (84,800 d.p.m./pmol) was determined by the same procedures, using about 364,000 d.p.m. per tube. Samples containing [^{3}H]-E_4 were counted in Scintiverse (Fisher Scientific Co.).

Evaluation of competition data; relative values of association constants. Since the amounts of E_2 used in these experiments were in large excess in relation to the available estrogen receptor, the sum of the concentration of receptor bound to E_2 ([E_2R]) and to the competitor ([CR]) was considered to equal the concentration of total receptor available, i.e.

$$[E_2R] + [CR] = [R_T]$$

where $[R_T]$ is the total concentration of receptor determined by incubations with [^{3}H]-E_2 in the absence of competitor.

As previously described [1], competition for a single set of binding sites implies that

$$\frac{K_{aC}}{K_{aE_2}} = \frac{[CR]}{[E_2R]}\frac{[E_2]}{[C]}$$

where K_{aC} and K_{aE_2} are the constants of association of C and E_2 to the receptor.

Under the experimental conditions used, in which the receptor is saturated by the ligands,

$$\frac{[CR]}{[E_2R]} = \frac{[R_T] - [E_2R]}{[E_2R]}$$

$$= \frac{1 - \text{fraction of } E_2 \text{ retained}}{\text{fraction of } E_2 \text{ retained}} = \frac{1-f}{f}$$

or

$$\frac{\text{Fraction of } E_2 \text{ displaced}}{\text{Fraction of } E_2 \text{ retained}} = \frac{1-f}{f}$$

$$= \frac{K_{aC}}{K_{aE_2}}\frac{[C]}{[E_2]} \simeq \frac{K_{aC}}{K_{aE_2}}\left(\frac{C}{E_2}\right)$$

where (C/E_2) is the ratio of concentrations of competitor and E_2 in the assay mixture.

A plot of $(1-f)/f$ vs (C/E_2) served to test the assumption of competition of E_4 or EE with E_2 for a single set of binding sites, which would require that a straight line were obtained. The slope of such a regression line would indicate the relative value of the constants of association of E_2 and the competitor to the receptor.

Cytosol binding saturation analysis, Scatchard plot. In order to determine association constants of E_2, E_4 and EE to estrogen receptors in cytosol and the maximum number of binding sites available, aliquots of cytosol were incubated separately with various amounts of each of the tritiated compounds. The procedure described above was used for the incubations, separation of bound and unbound ("free") steroid, and correction for non-specific binding. The amount of high specific activity labeled estrogens used ranged from 42,400 to 424,000 d.p.m. (0.40–4.0 pmol) for E_2, 39,400 to 394,000 d.p.m. (0.34–3.4 pmol) for E_4, and 42,000 to 424,000 d.p.m. (0.37–3.7 pmol) for EE. The ratios of specifically bound to unbound labeled estrogen (B/F) and the corresponding concentrations of receptor-bound ligand (B, as pmol/ml of assay solution) were plotted in linear coordinates. Binding of the estrogen to a set of sites characterized by a constant of association K_a, would result in a linear function $B/F = -K_aB + nK_a$ where n is the concentration of specific binding sites. The slope of this line corresponds to $-K_a$ and the intercept with the abscissa ($B/F = 0$) corresponds to n. This value can be referred to the protein content in the assay solution and is usually expressed as fmol of ligand/mg cytosol protein.

RESULTS

Table 1 presents the results of experiments of competition of E_4 and E_2, or EE and E_2, for saturable

Table 1. Competition

	E_4/E_2 (mol/mol)	E_2 displaced (%)	$(1-f)/f$
	E_4/E_2 (mol/mol)	E_2 displaced (%)	$(1-f)/f$
	E_4/E_2 (mol/mol)	E_2 displaced (%)	$(1-f)/f$
	E_4/E_2 (mol/mol)	E_2 displaced (%)	$(1-f)/f$
	EE/E_2 (mol/mol)	E_2 displaced (%)	$(1-f)/f$
	EE/E_2 (mol/mol)	E_2 displaced (%)	$(1-f)/f$

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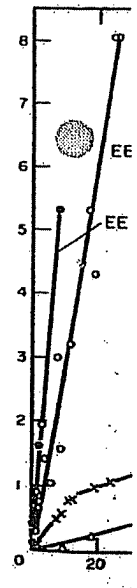

1. Competition of
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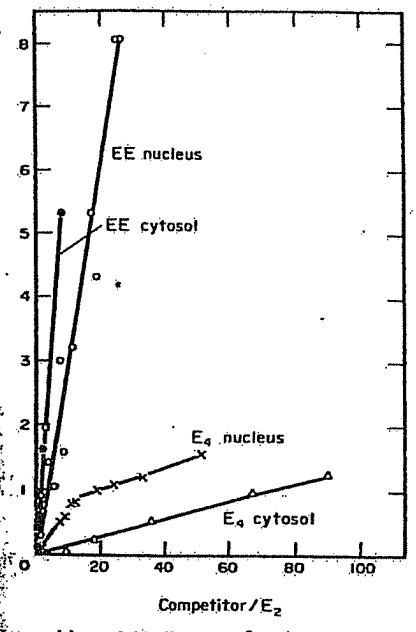
trium by estetrol

Table 1. Competition of estetrol (E_4) and ethynylestradiol (EE) with estradiol (E_2) for binding to receptors in cytosol of proliferative human endometrium

Competition data							Total receptor concentration (fmol/ml protein)	Regression line $\frac{1-f}{f} = a\frac{C}{E_2} + b$ a	b	Correlation coefficient	$\frac{K_a}{K_4}$
E_4/E_2 (mol/mol)	4.5	9	13	18	45	89					
E_2 displaced (%)	4	10	14	20	31	49	190	0.011	0.016	0.99	95
$(1-f)/f$	0.04	0.11	0.16	0.25	0.45	0.96					
E_4/E_2 (mol/mol)	9	18	45	89	180						
E_2 displaced (%)	20	27	51	65	77		230	0.018	0.130	0.99	55
$(1-f)/f$	0.25	0.37	1.0	1.9	3.3						
E_4/E_2 (mol/mol)	9	18	36	67	89	180					
E_2 displaced (%)	5	18	34	48	55	61	81	0.020	0.048	0.99	50
$(1-f)/f$	0.05	0.22	0.52	0.92	1.2	2.1					
E_4/E_2 (mol/mol)	9	18	45	89							
E_2 displaced (%)	18	23	46	61			80	0.016	0.350	0.97	60
$(1-f)/f$	0.22	0.3	0.85	1.6							
EE/E_2 (mol/mol)	0.18	0.46	0.92	1.8							
E_2 displaced (%)	10	29	38	62			80	0.902	−0.078	0.98	1.1
$(1-f)/f$	0.11	0.40	0.61	1.6							
EE/E_2 (mol/mol)	0.23	0.46	0.92	1.8							
E_2 displaced (%)	12	16	34	49			55	0.542	−0.010	0.99	1.8
$(1-f)/f$	0.14	0.19	0.52	0.96							

ing in endometrial cytosol. Each experiment included assays conducted at various relative ratios of labeled competitor and [^{3}H]-E_2 using aliquots of cytosol from a single specimen of proliferative tissue. Concentrations of [^{3}H]-E_2 were maintained constant and above the levels needed for saturation of the receptor present in the sample. The total receptor concentration in each cytosol preparation, expressed in fmol/mg protein and shown in the table, was determined by measuring the binding of [^{3}H]-E_2 in the absence of competitor. This value was taken as unity and the amount of specifically bound [^{3}H]-E_2 in the presence of competitor was expressed as "fraction of E_2 retained" (f). As described above, $(1-f)/f$ should be proportional to the ratio of concentrations of competitor and E_2 if these compounds compete for a single set of binding sites. The table shows the values of slopes and intercepts of the regression lines best fitting the experimental points by the least-square method. The correlation coefficients indicate linearity and, consequently, competition of E_2, EE and E_4 for a single set of binding sites. The relative values of association constants of E_2 and E_4 to the receptor were in the range of 50–95 with an average of 65. In contrast, K_{aE_2} was only 1.1–1.8 times greater than K_{aEE}.

The regression lines corresponding to experiments 3 and 4b are shown in Fig. 1. This figure includes, for purposes of comparison, data previously reported [1], obtained by incubations of endometrial



Competition of binding of [^{3}H]-E_2 to estrogen receptors in nuclei and cytosol of human proliferative endometrium by estetrol (E_4) and ethynylestradiol (EE).

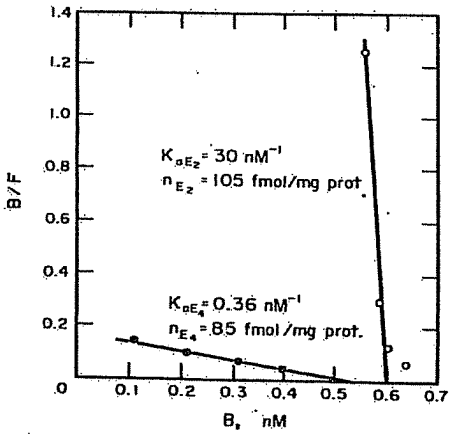


Fig. 2. Specific binding of estradiol (E_2) and estradiol (E_4) in endometrium cytosol.

slices with mixtures of [^{3}H]-E_2 and E_4 or [^{3}H]-E_2 and EE and measurement of nuclear concentrations of specifically bound [^{3}H]-E_2. Lack of linearity is noted only in the nuclear data corresponding to the competition between E_2 and E_4.

The results presented in Table 1 and Fig. 1 can be interpreted to indicate that E_2, EE and E_4 bind to the same set of sites in endometrial cytosol with relative association constants of 100:70:1.5. Specific binding of E_2 and EE in the nucleus also appears to reflect competition for a single set of sites. In contrast, competition of E_2 and E_4 for nuclear binding appears to reflect heterogeneity in nuclear sites, viz. the relative association constants of E_2 and E_4 are 100:16 for about 65% of the sites and 100:1.5 for the other 35%.

The association constants of cytosol receptors, determined by analyses of Scatchard plots from several endometrial specimens, varied from 2 to 30 nM^{-1}. Figure 2 presents the results of one experiment in which binding of [^{3}H]-E_2 and [^{3}H]-E_4 was studied in different aliquots of cytosol of the same endometrial specimen. The ratio of association constants of cytosol receptor for E_2 and E_4 in this example was 100:1.2 ($K_{aE_2} = 30\ nM^{-1}$, $K_{aE_4} = 0.36\ nM^{-1}$). The concentration of binding sites in cytosol were about 100 fmol/mg protein, 20% lower for E_4 than for E_2. Similar agreement was found in the other three experiments. Scatchard plots of data on binding of E_2 and EE to receptors in aliquots of cytosol samples used in experiment 5 (Table 1) indicated a ratio $K_{aE_2}/K_{aEE} = 1.3$ and total concentrations of binding sites of 55 and 63 fmol/mg protein for E_2 and EE, respectively.

DISCUSSION

The heterogeneity of estradiol binding in the nuclei of uterine tissue has been previously suggested on the basis of results from experiments in which the labeled steroid was extracted from nuclear preparations with salt solutions of various concentrations [2] or in the presence of intercalating agents [4], although the significance of the results from differential extraction experiments has been questioned [5]. Differences in the extractability of E_2 and nafoxidine were also interpreted to reflect heterogeneity in nuclear binding sites [4].

The results from the series of experiments reported here are consistent with binding of E_2, E_4, and EE to a single set of sites in human endometrial cytosol. Therefore, the heterogeneity we have observed during competition of E_2 and E_4 for nuclear binding does not seem to be due to heterogeneity in cytosol receptors.

It is of interest to note that the competition of E_4 for binding of E_2 appears to be 10 times more effective in nuclei than in cytosol when measured cellular E_4/E_2 concentration ratios be association constants 100:16 from nuclear 100:1.5 from cytosol data). This finding a difference in binding properties of the the receptor in cytosol and to the chromatin acceptor sites. In contrast, the competition curve for E_2 and E_4 in cyto different from the slope of the competition nuclear binding at intracellular E_4/E_2 than 15. The relative values of the associ stants of E_2 and EE are 1.5 in cytosol and 3 this difference, however, may not be statistically nificant.

The two slopes in the curve of competition and E_4 for nuclear binding differ by a factor (Fig. 1). The existence of these two types of sites in the nucleus may reflect different receptor configurations or different locations complexes on the chromatin.

Martucci and Fishman [6] reported lack of tropic activity of E_4, a finding that would support the possibility that the nuclear receptors which are hardly exchangeable with be responsible for biological action. Chang published) failed to see estrogenic or anti actions of E_4 injected in rats at levels of for 4 days, as evaluated by the rate of transport. On the other hand, Holinka and (Biol. Reprod., in press) could detect effects uterine weight and alkaline phosphatase acti high doses of E_4 (10–50 μg/day) were inje immature rats for 1 or 2 days.

Acknowledgements—This investigation was supported grant HD 07197, awarded by the National Health.

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EXHIBIT "D"

Uterine vascular effects of estetrol in nonpregnant ewes

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Estetrol is produced by the fetal liver and has been suggested to be a sensitive indicator of fetal well-being. Although the uterine vascular effects of estrogens (17β-estradiol, estriol, and estrone) have been extensively investigated in our laboratory and those of others, the ability of estetrol to dilate the ovine uterine vasculature is not presently known. The present experiment was designed to compare the vasoactivity of estetrol to that of a second pregnancy-associated estrogen, estriol. Five nonpregnant oophorectomized ewes were chronically instrumented with catheters in the femoral artery, femoral vein, uterine arteries, and electromagnetic flow probes on both uterine arteries. Upon recovering from operation, animals received unilateral intra-arterial (uterine) injections of either estriol (0.1, 0.3, 1, and 3 μg) or estetrol (1, 3, 10, and 30 μg). Ewes received only one dose of either estetrol or estriol daily and all doses were given in a randomized order. Uterine blood flow responses were continuously monitored and the time of onset, peak, and duration were recorded. The time of onset (38 ± 2 minutes), time of peak response (75 ± 1 minute), and duration (189 ± 7 minutes) were approximately equal to those observed for estriol. On the basis of the data obtained in the present study we have determined that estetrol is 15 to 30 times less potent than estriol as a uterine vasodilator. (Am. J. Obstet. Gynecol. 148:735, 1984.)

The effects of estrogen on the uterine vasculature have been investigated in several different species.[1-3] In recent years these studies have been largely conducted in nonpregnant oophorectomized ewes.[4-6] Upon administration, estrogen produces a profound uterine vasodilation which occurs after an initial delay of 35 to 45 minutes. This increase in uterine blood flow peaks at approximately 75 to 120 minutes and returns to control levels by 4 hours. In the nonpregnant ovine, 17β-estradiol and estriol are approximately equivalent as uterine vasodilators.[5, 13] In recent years a fetus-derived estrogen, estetrol (15α-hydroxyestriol) has been used by several investigators[7-10] to assess fetal well-being. The physiologic role of estetrol is unclear, but potent estrogens of fetal origin could be important in dilating the uterine and placental vasculature. The purpose of this study was to compare the uterine vasodilator effects of estetrol to those previously reported for estriol in the nonpregnant ovine model.

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Material and methods

Five nonpregnant oophorectomized ewes of mixed breed, weighing between 42 and 60 kg, were used in this study. Ewes were sedated with diazepam (10 mg intravenously), and with the ewes under further sedation (thiopental, 250 mg intravenously), a hyperbaric spinal anesthetic was administered (Pontocaine hydrochloride, 12 mg). The maternal femoral artery and vein were cannulated with polyvinyl catheters. The uterine arteries were fitted with electromagnetic flow probes (3.5 or 4.0 mm in diameter), and a lateral branch of each artery was catheterized with a polyvinyl catheter (0.40 by 0.70 inch) to allow direct intra-arterial administration of the estrogens to be tested. Ewes were castrated to prevent cyclic changes in the hormonal milieu. Catheters and flow probe cables were passed subcutaneously to the left flank of the sheep and placed in a cloth pouch secured to the side of the ewes. All catheters were filled with heparin (1,000 U/ml) to maintain patency.

After operation, the animals were placed in portable cages and received water and commercial feed ad libitum. Ewes were allowed to recover from operation for 5 to 7 days prior to testing. The ewes received an intravenous injection of 17β-estradiol (1 μg/kg) each evening to prevent uterine atrophy, which would occur because of castration. Uterine arterial blood flow was monitored with a square-wave electromagnetic flowmeter (Dienco RF-1000, Los Angeles, California). Electromagnetic flow probes were calibrated with saline prior to implantation and were linear over the range of flow measured. Flowmeters were equipped with electronic zeroes which were verified as being accurate dur-

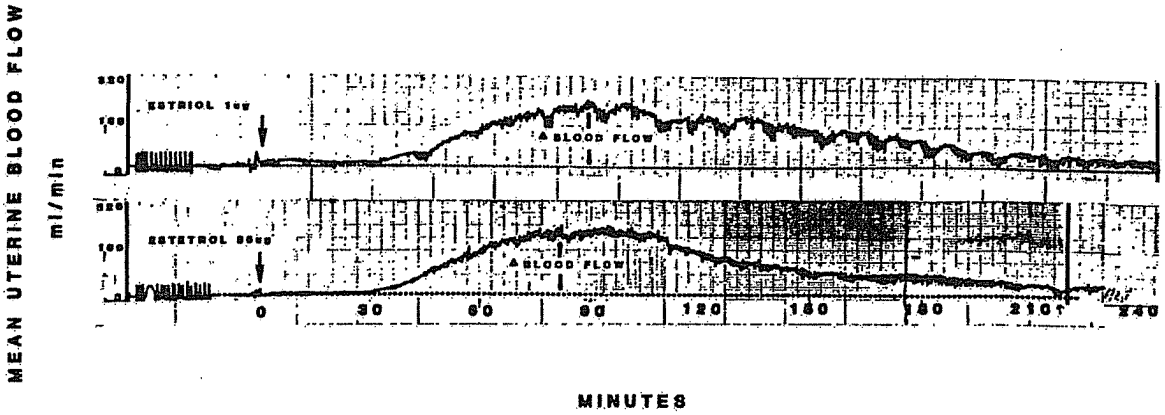

Fig. 1. The uterine vascular response to intra-arterial injections of estriol (1 μg) and estetrol (30 μg). Estriol and estetrol were given into the right uterine artery of the same ewe on 2 different days.

ing vascular occlusion. Blood pressure was monitored by a Micron (MP 15) pressure transducer, and heart rate was determined by a Beckman cardiotachometer. All parameters were continously recorded on a pen-writing recorder (Beckman Dynograph R-612). Differences between values were determined with Student's t test with significance defined as $p < 0.05$.

Experimental protocol. A stock solution of estriol (Sigma Chemical Company, St. Louis, Missouri) and estetrol (Steraloids, Inc., Wilton, New Hampshire), was prepared by dissolving the estrogens in ethanol and diluting the stock solution in sterile 0.9% saline. Solutions for intra-arterial bolus injections were prepared in concentrations of 0.1, 0.3, 1.0, and 3.0 μg/ml for estriol and 1.0, 3.0, 10.0, and 30.0 μg/ml for estetrol. The final ethanol concentration was 0.1% by volume, and no vasodilating effect of the vehicle was noted. The doses of estrogen were given in a randomized order, with only one intra-arterial injection of either estriol or estetrol administered each day. Blood flow to the contralateral horn was monitored and did not change in these experiments. Uterine blood flow responses were continuously monitored and the time of onset, peak, and duration were recorded.

Results

The uterine vascular response to the intrauterine administration of estriol and estetrol in one ewe on two different days is illustrated in Fig. 1. The response to the intra-arterial injection of either estriol or estetrol has the same basic characteristics as the reponse to 17β-estradiol: (1) a delay of approximately 35 to 45 minutes between intra-arterial injection and beginning of the response, (2) a peak response at 80 to 120 minutes, and (3) a similar time interval for return to baseline (170 to 240 minutes).

Forty experiments were performed on five ewes for analysis of the vascular responses to varying doses of estriol and estetrol. The dose-response curves depicted as the absolute change in uterine blood flow in milliliters per minute are illustrated in Fig. 2. The maximum change in uterine blood flow for estriol was elicited at a dose of 1 μg/ml (123 ± 19 ml/min, mean ± SEM), whereas estetrol required 30 μg/ml to attain a similar response (130 ± 20 ml/min, mean ± SEM). Any further increase in the dosage of estriol or estetrol did not increase the magnitude of the response significantly. Although estetrol produces uterine vasodilation equal in magnitude to that of estriol, it is approximately 15 to 30 times less potent on an equal weight basis. The characteristics of the uterine vascular response to estriol and estetrol for varying doses of the estrogens (micrograms) are shown in Table I. The mean onset of the vascular response was delayed 39 ± 2 minutes for estriol and 38 ± 2 minutes for estetrol, whereas the peak response occurred at 81 ± 2 minutes for estriol and 75 ± 1 minute for estetrol. These values were not significantly different. The duration of the response to estriol was 241 ± 11 minutes, while the duration of response to estetrol was 189 ± 7 minutes. This difference was statistically significant ($p < 0.05$).

Comment

The placenta as an organ of fetal exchange is essential in the maintenance of fetal homeostasis. Uterine placental blood flow is the single most important component of placental exchange and any alteration could have an adverse effect on the fetus. During pregnancy, blood flow to the uterus is markedly increased. This increase in blood flow is due to both vessel growth and local vessel vasodilatation. The blood vessels supplying the placenta progressively dilate, achieving a state in which minimal or no further dilatation can occur.[11] Estrogens are known to be potent uterine vasodilators,

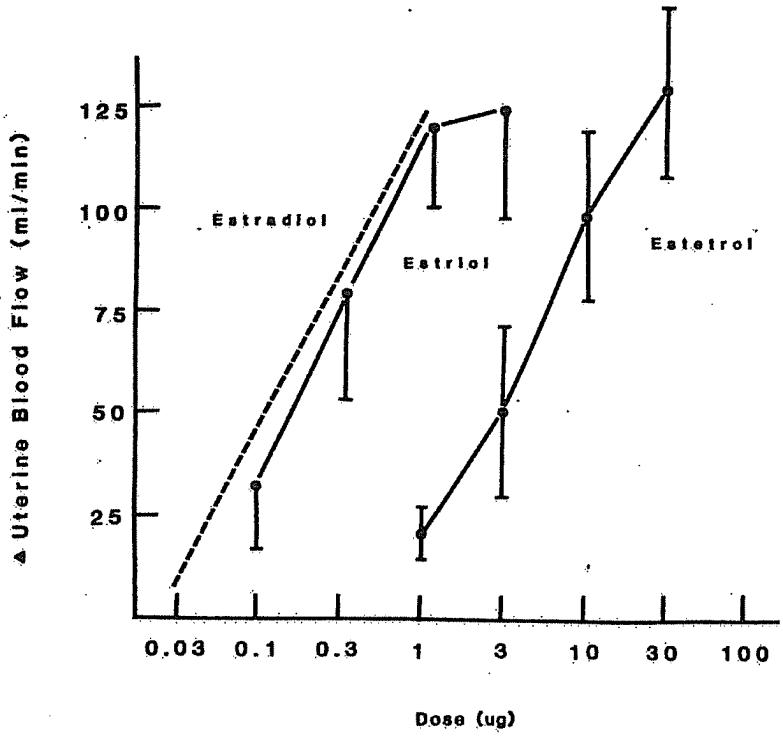

Fig. 2. Uterine vascular effects of estriol and estetrol, depicted as the change in uterine blood flow (milliliters per minute) versus dose of estriol and estetrol in micrograms administered. The *dashed line* represents previously obtained data for 17β-estradiol.[13]

and during pregnancy fetally and placentally produced estrogens could be important in the maintenance of uteroplacental blood flow. In the second half of gestation, the production of estriol involves both the fetal and the placental units.[12] Recently a more polar estrogen, estetrol (15α-hydroxyestriol), has been described as a steroid which is almost exclusively produced by the fetus.[7–10] Since the fetus is one of the main sources of estriol and the major source of estetrol, it is conceivable that the fetus could be partially responsible for maintaining uteroplacental blood flow by modulating vascular tone.

In the present study, we have demonstrated that the intra-arterial administration of estetrol produces uterine vasodilatation in the nonpregnant oophorectomized sheep. Estetrol was approximately 15 to 30 times less potent than estriol in dilating the uterine vasculature of the nonpregnant ewe. However, the vascular response to estetrol was qualitatively similar in magnitude and duration to that of the other estrogens previously studied in our laboratory and by others.[4–6, 13] Previous studies have also shown that estriol and estradiol are approximately equipotent as uterine vasodilators in this animal model.[5, 13] Thus the present data suggest that local uterine and placental concentrations of estetrol would have to be 15 to 30 times higher than either estriol or 17β-estradiol to be important in dilating the uteroplacental vasculature during pregnancy. In 1974, Sciarra and co-workers[7] showed that maternal circulating levels of estetrol are approximately 10 times less than those observed for estriol. Thus, on the basis of their report and our data it appears unlikely that estetrol occurs, in concentrations high enough to play a major role in dilating the uteroplacental vasculature.

In the ovine model, most estrogens have similar vas-

Table I. Time of onset, peak, and duration of response to varying doses of estriol and estetrol

Dose (μg)	*Onset (min)*	*Peak (min)*	*Duration (min)*
Estriol			
0.1	39 ± 5	74 ± 3	210 ± 12
0.3	44 ± 2	82 ± 2	263 ± 7
1.0	38 ± 1	85 ± 2	243 ± 10
3.0	36 ± 4	83 ± 7	247 ± 27
Estetrol			
1.0	42 ± 1	74 ± 3	184 ± 19
3.0	35 ± 4	73 ± 5	170 ± 17
10.0	38 ± 3	75 ± 2	198 ± 14
30.0	35 ± 1	76 ± 3	203 ± 11

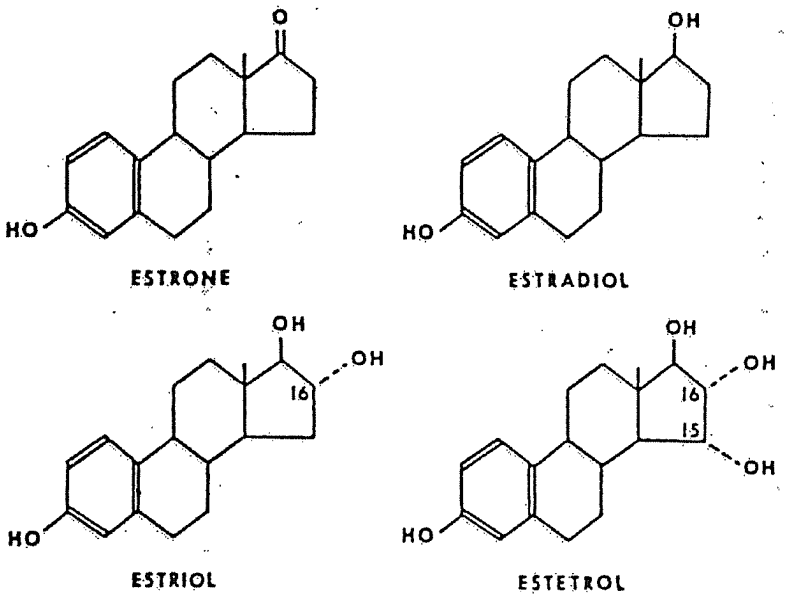

Fig. 3. The structural formulas of estrone, estradiol, estriol, and estetrol are shown. Note that the only structural difference between estriol and estetrol is a hydroxy group at the carbon 15 position.

cular responses (onset, peak, and duration) but have different biologic potencies. These differences in potency may be explained by the ability of the compound to bind to the common estrogen cytoplasmic receptors. The receptor steroid complex, once formed, is then transferred to the nucleus and a biologic response results. The structures of 17β-estradiol, estrone, estriol, and estetrol are illustrated in Fig. 3. It is interesting to note that the addition of hydroxy group at the carbon 15 position of estriol greatly reduces its biologic potency. This reduction in potency may be due to the altered polarity or molecular configuration of the compound. Since estetrol has hydroxy groups at carbons 15 and 16, unlike 17β-estradiol and estriol, a greater concentration may be needed to reach and bind with the receptor. This may be due to increased stearic hindrance at the estrogen receptor site. Since all estrogens compete for a common receptor site, it seems reasonable to assume that the vasodilator potencies observed in the nonpregnant ovine may also be extrapolated to other species.

We wish to thank June Austin for her technical assistance.

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EXHIBIT "E"

BIOLOGY OF REPRODUCTION 22, 913–926 (1980)

Comparison of Effects of Estetrol and Tamoxifen with Those of Estriol and Estradiol on the Immature Rat Uterus[1]

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ABSTRACT

The estrogenic effects on this immature rat uterus of estetrol [1,3,5(10)-estratriene-3,15α, 16α, 17β-tetrol] (E_4), a major metabolite of placental estrogens in the human fetus, and of tamoxifen (TAM), an antiestrogen, were compared with those of estradiol (E_2) and estriol (E_3). The compounds were injected s.c. daily, for 3 days, into 22- or 23-day-old Sprague-Dawley rats at a dose of 50 μg/100 g BW of E_4 or TAM, and 1 μg/100 g BW of E_2 or E_3. Tissue was collected 24 to 27 h after the third injection.

Both E_4 and TAM increased uterine cytosol progesterone receptor (PR) content and PR/DNA. Progesterone receptor levels per mg DNA were 1.7, 2.8 and 5.1 pmol in the control, E_4 and TAM groups, respectively. Both compounds showed estrogenic activity in raising uterine wet weight and content of total and soluble (cytosol) protein. Tamoxifen augmented DNA content but the effect of E_4 on this parameter was statistically significant in only one of two series of experiments. Specific induction of soluble protein, assessed by the ratio cytosol/total protein, was noted after E_2 or E_3 injection, but not after treatment with E_4 or TAM.

Evaluation of morphologic changes in luminal and glandular epithelium by light and electron microscopy indicated enhanced synthetic and secretory activity after administration of all four compounds. Estradiol and TAM changed the shape of luminal epithelial cells from cuboidal to tall columnar, developed prominent Golgi systems and rough endoplasmic reticulum, and promoted the formation of secretory granules. Similar but less pronounced ultrastructural changes were observed after E_3 or E_4 treatment: the height of the luminal columnar epithelium was lower, the microvilli were shorter and the secretory granules were absent. The ultrastructural changes in glandular epithelium paralleled those found in the luminal epithelium. Cell degeneration was observed in luminal and glandular epithelium after E_3 or E_4 treatment and, to a small degree, after TAM treatment. Estradiol produced this effect in glandular but not in luminal epithelium. The phenolic steroids, but not TAM, increased the number of uterine eosinophils significantly. TAM raised the endometrial/myometrial ratio of cross-sectional areas significantly, whereas E_2, E_3 or E_4 showed a tendency to lower those ratios.

In general, E_2 treatment promoted the most marked changes, followed by TAM, E_3 and E_4. On the basis of the present biochemical and morphologic results, it is concluded that E_4 and TAM have estrogenic effects on the immature rat uterus. However, the estrogenic potency of E_4 relative to E_2 or E_3 was low at the dosage and timing of administration used in these experiments; effects of E_4 introduced into the circulation at a constant rate were not evaluated. These results suggest that the conversion of E_2 to E_4 in the human fetus might represent an efficient mechanism of inactivation of the placental hormone.

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INTRODUCTION

Estetrol (E_4) is a major estrogen metabolite in the human fetus (Hagen et al., 1965; Gurpide et al., 1966; Zucconi et al., 1967) and the question therefore arises whether the compound has estrogenic properties or is an inactive metabolic end product of estradiol (E_2) and thus represents a fetal metabolic pathway to neutralize the estrogenic action of E_2. Estetrol

was found to bind to estrogen receptors in the rat uterus (Martucci and Fishman, 1976) and in human endometrium (Tseng and Gurpide, 1976, 1978) with an association constant much lower than that of E_2.

In the present study, the estrogenic activity of E_4 was evaluated by its action on the immature rat uterus in relation to that of E_2, estriol (E_3) and tamoxifen (TAM), compounds which differ not only in their potency but also in their residence time on nuclear binding sites (Anderson et al., 1975; Jordan et al., 1977; Koseki et al., 1977a).

Changes in several biochemical parameters of the immature rat uterus in response to treatment with E_4 were described in a previous publication (Holinka and Gurpide, 1979). Uterine wet weight and luminal fluid content were elevated at 6 h after a single s.c. injection of E_4 (50 μg/100 g BW); protein content and alkaline phosphatase activity were increased at 48 h following administration of a second dose of E_4 at 24 h, but DNA content was not significantly raised at that time. These effects were much smaller than those produced by estradiol at a dose of 1 μg/100 g BW, similarly administered; the effects were also smaller, although not statistically different, from those obtained with E_3, also at a dose of 1 μg/100 g BW.

The purpose of the present series of experiments was to extend these studies as follows: 1) to compare the effects of E_2, E_3 and E_4 with those of TAM; 2) to prolong the administration of the compounds to 3 days to determine whether E_4 and TAM are able to increase uterine DNA content; 3) to test the ability of these compounds to increase progesterone receptor levels; 4), to distinguish between effects on total and cytosol (soluble) protein content; 5) to evaluate differential effects on luminal and glandular epithelium by light and electron microscopy; and 6) to compare by light microscopy the changes in the cross-sectional areas of myometrium and endometrium.

MATERIALS AND METHODS

This study involved two series of experiments. Series A was designed to determine the uterine response to three daily injections of E_2, E_3, E_4 and TAM by measurement of the following parameters 24 to 27 h after the third injection: DNA and total protein, both assayed in whole homogenate, wet weight and luminal fluid. Subsequently, series B was designed to determine the ability of the same compounds, on an identical injection schedule, to induce cytosol progesterone receptors. Soluble (cytosol) protein and DNA were measured in the supernatant and pellet, respectively, after 1 h centrifugation at 105,000 × g.

Animals

Immature Sprague-Dawley rats were purchased from Zivic-Miller Labs., Inc., Allison Park, PA, and housed in pairs in a room used exclusively for these animals, which was kept at 21–22°C and on a 12L:12D cycle. Purina laboratory chow and tap water were freely available. Injections were started at 22 or 23 days of age.

Chemicals and Buffers

Crystalline E_2, E_3, progesterone (P) and cortisol (F) were obtained from Steraloids, Wilton, NH. tamoxifen (free base) was a gift from Stuart Pharmaceuticals (Division of ICI United States, Inc.), Wilmington, DE, and E_4 was purchased from the Lamar Research Group (currently Synsteroids, Konstanz, West Germany). The purity of E_4 was verified by high pressure liquid chromatography and gas chromatography-mass spectroscopy, as reported elsewhere in greater detail (Holinka and Gurpide, 1979). Each 50 μg dose of E_4 was found to contain less than 5 ng E_2 or E_3. Therefore, the maximum amount of E_2 or E_3 that might have been introduced with each injection was less than 3 ng, a dose which we do not consider sufficient to explain the E_4 effects reported in this study. The radiochemical purity of [1,2-^{3}H]-P (sp act 55.7 Ci/mmol; New England Nuclear, Boston, MA) was verified by chromatography prior to use.

The buffer used in experimental series A was 10 mM Tris (Sigma), pH 7.4; in series B, 10 mM Tris, pH 7.4, containing 1 mM EDTA (Fisher Scientific), 1 mM dithiothreitol (Calbiochem), 0.01% sodium azide (Eastman Kodak) and 30% v/v glycerol (Fisher Scientific). This buffer was also employed in the preparation of the dextran-coated charcoal (DCC) suspension (0.5% Norit A, 0.05% Dextran T70, Sigma) used for the progesterone receptor assay.

Treatment, Tissue Collection and Storage

Estradiol, E_3, E_4 and TAM were dissolved in ethanol, the solvent was evaporated after addition of 0.25 ml propylene glycol and the residue was dissolved in 25 ml arachis oil (Planter's peanut oil) to obtain a concentration of 2 μg/ml for E_2 and E_3, and of 100 μg/ml for E_4 and TAM. The compounds were administered s.c. under the dorsal skin in the following amounts per 100 g BW: 1 μg E_2, 1 μg E_3, 50 μg E_4 and 50 μg TAM. Control rats received 0.5 ml of the vehicle. Injections were given daily, between 1000–1400 h. The animals were killed by cervical dislocation between 24–27 h after the third injection. The uteri used for biochemical studies were cleaned of fat and connective tissue, blotted with 0.9% saline-dampened filter paper and weighed. Small transverse cuts were subsequently made along each uterine horn at ∿2 mm intervals and the luminal fluid was removed by gentle squeezing between saline-dampened filter paper. The uteri were reweighed to obtain values for wet weight and, by difference, for luminal fluid content. The tissue was then either stored at −30°C for DNA and protein assays (series A) or used fresh for P

receptor determinations (series B). The nuclear pellet and cytosol aliquots of series B were kept frozen at −30°C for DNA and protein determinations.

Progesterone Receptor (PR) Assay

The PR assay was adapted from a protocol used by Feil et al. (1972) for the mouse and rat uterus. After brief storage in saline on ice, the uteri were minced and gently homogenized in a glass/glass homogenizer at a tissue/buffer ratio of 37.5 mg/ml. All subsequent steps were done at 0–4°C. Cytosol (0.2 ml), obtained by centrifuging at 105,000 × g for 1 h, was incubated in triplicate in 3.2×10^{-9} M [^{3}H]-progesterone ([^{3}H]-P) plus 3.2×10^{-7} M cortisol (F) to determine total binding and in 3.2×10^{-9} M [^{3}H]-P plus 3.2×10^{-7} M F plus 3.2×10^{-7} M P, to assess nonsaturable binding. Steroids were dissolved in ethanol and kept in 5% ethanol-buffer solution. Fifty microliters of that solution and, after 90–120 min of incubation, 0.2 ml of DCC were added to each vial, which subsequently was gently vortexed and placed on a rotating apparatus for 8–10 min and then centrifuged 10 min at 800 × g. Aliquots (0.3 ml) of the supernatant were counted in 10 ml Dimiscint (National Diagnostics) at an efficiency of 48%.

The tissue/buffer ratio used in this study corresponded to 1.1–1.9 mg protein/ml cytosol. The measured amounts of receptor-bound P were shown to be proportional to the protein concentration within that range.

Three independent experiments testing the approach to equilibrium over a period from 2.5 to 180 min of incubation showed maximum binding at 2 h, which remained stable to at least 3 h. At 1 h, over 90% of the maximum was reached.

An experiment to check the effect of length of incubation showed that specific binding remained within 5% between 5–15 min after addition of DCC.

Dissociation constant values, obtained by Scatchard analysis, ranged from 5.5×10^{-8} to 1.3×10^{-9} M.

DNA and Protein Assays

In experimental series A, defrosted tissue was homogenized at a tissue/buffer ratio of 20 mg/ml and quadruplicate aliquots were taken for DNA determination (Burton, 1956). The homogenate was again frozen at −30°C for protein assays (Lowry et al., 1951). In series B, triplicate aliquots for DNA and soluble protein were taken from the rehomogenized pellet and the stored cytosol, respectively. Calf thymus DNA (Sigma) and bovine serum albumin (Sigma) served as standards.

Preparation of Tissue for Morphologic Studies

Uteri were removed from 3 rats in each treatment group. The left horn was placed in 10% formalin overnight, then divided transversely into 4 pieces and embedded in paraffin for use with light microscopy. Cross sections from different levels were stained with hematoxylin and eosin or with aldehyde fuchsin. The endometrial/myometrial ratios of the cross-sectional areas were estimated from tissue stained with Masson's trichrome to enhance contrast between the two regions (Luna, 1968). An average of 13 cross sections in each group were photographed and the areas corresponding to the endometrium and myometrium were cut and weighed. Tissue pieces ~1 mm^3 for electron microscopy were obtained from the middle third of the right horn of each of the 3 animals in each treatment group. They were fixed in 0.1 M phosphate buffered 4% glutaraldehyde pH 7.4, for 4 h, fixed further in buffered 1% OsO_4, dehydrated in a graded series of ethanol solutions and embedded in Epon (Luft, 1961). Silver to grey thin sections were stained with uranyl acetate and lead citrate, examined and photographed in a Hitachi HS-8 electron microscope.

RESULTS

Figure 1 presents progesterone receptor (PR) levels 24–27 h after the last of three injections of E_2, E_3, E_4 or TAM given at 24 h intervals. All four compounds produced highly significant increases in PR concentrations relative to DNA (Fig. 1A). A similar pattern emerged when PR levels were expressed per total protein (Table 1). Estradiol produced the highest elevation, followed by TAM, E_3 and E_4; at comparable dosage, E_2 was more effective than E_3, and TAM was more effective than E_4. However, when the PR levels were expressed as pmol specifically-bound progesterone/mg cytosol protein (Fig. 1B), a quite different pattern of responses was noted. Progesterone receptors/mg cytosol protein after TAM treatment became comparable to those after E_2 injections, largely due to the selective stimulation of soluble protein synthesis by E_2 but not by TAM. As shown in Fig. 2, all compounds increased total as well as soluble proteins (mg protein/mg DNA) in the cell, but only E_2 and E_3 raised the ratio of soluble to total protein, as indicated on top of the figure. From this it can be concluded that E_2 and E_3 but not E_4 or TAM can promote the selective increase of all or some of the soluble proteins present in the cytosol fraction at the pH and ionic strength of this buffer.

Uterine wet weight increased significantly in all treatment groups as a result of both hypertrophy, shown by the gain in wet weight/DNA ratio, and hyperplasia, shown by the increase in DNA content (Table 1). In agreement with results reported by Lerner (1964) in relation to the effects of E_2 on the immature rat uterus, the total protein/wet weight ratios remained essentially unchanged by all treatments (Table 1).

Evaluation of the uterotropic effects of estrogens by measurement of amounts of DNA per uterus (Table 1) revealed clear changes after injection of E_2 and E_3 (~90 and 40% increases, respectively). Estetrol and TAM, administered at doses 50-fold higher than those of E_2 and E_3, also provoked elevations in

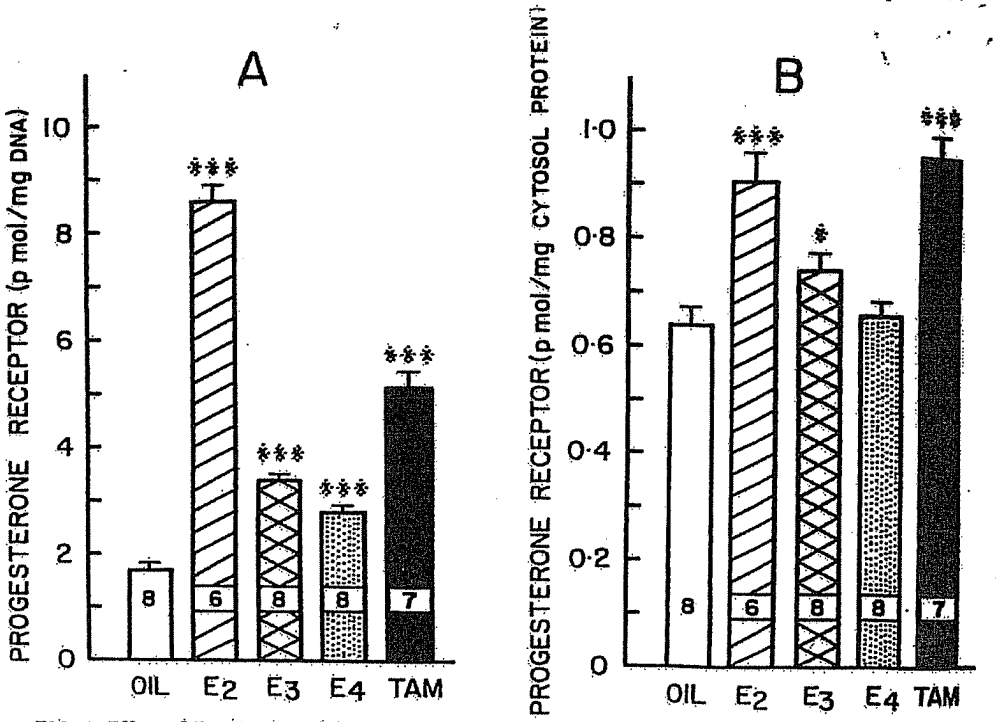


FIG. 1. Effect of E_2, E_3, E_4 or TAM after 3 daily injections, administered at 24 h intervals, on progesterone receptor levels in the immature rat uterus, expressed A) per mg DNA and B) per mg cytosol protein. Amounts of compounds, injected s.c. per 100 g BW: E_2, 1 μg; E_3, 1 μg; E_4, 50 μg; TAM, 50 μg; control, 0.5 ml arachis oil. The animals were killed 24–27 h after the last injection. Progesterone receptor levels were determined in individual uteri by cytosol (105,000 X g, for 1 h) incubation, in triplicate, with 3.2×10^{-9} M [^{3}H]-progesterone + 3.2×10^{-7} M cortisol, in the presence or absence of 100-fold progesterone; bound and free steroids were separated by dextran-coated charcoal. The number of rats in each treatment group appears as an insert in each bar.

Significance levels (Student's two-tailed t test) of differences from controls: ***($P<0.001$); **$P<0.01$; *$P<0.05$). Significance: A) E_3 vs E_4: $P<0.05$; E_3 vs TAM: $P<0.001$. B) E_3 vs E_4 NS; E_3 vs TAM: $P<0.001$.

DNA content, but the increases were smaller (~20 and 30%, respectively). Of the three series of experiments carried out to determine whether E_4 could increase the amount of DNA per uterus [two in the present study and one already published (Holinka and Gurpide, 1979)], statistically significant differences from the control groups were obtained only in series B, in which the experimental conditions for the measurement of DNA produced an extremely low intraassay variance.

Figure 3 illustrates values of parameters after 1, 2 or 3 daily estrogen injections. This figure includes previously published data (Holinka and Gurpide, 1979) on effects of E_2, E_3 and E_4, 24 or 36 h after a single injection, and 24 h after the last of 2 daily injections. Control values were nearly identical in all experimental conditions.

The effects of E_2 on uterine wet weight (Fig. 3A) and luminal fluid accumulation (Fig. 3B) differed from those of E_3 and E_4 not only in their magnitude, but also in the persistence of the rates of increase after a third injection.

Figures 3C and 3D show uterine DNA and total protein content, respectively. In the E_2-treated group, both parameters were markedly elevated at 48 h after the first of 2 injections. This increase occurred mainly as a result of the first injection and was not primarily

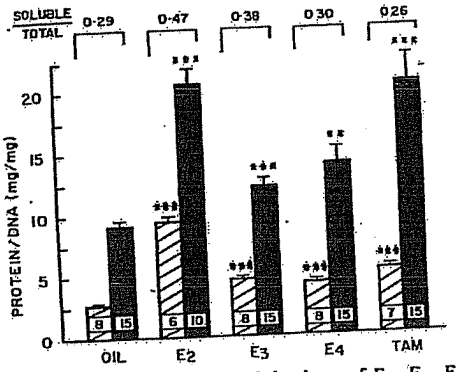


FIG. 2. Effect of 3 daily injections of E_2, E_3, E_4 or TAM on total protein, determined on whole homogenate (black bars) and soluble protein, from the 105,000 X g supernatant (hatched bars). Ratios soluble/total protein are given in the top panel. For details regarding injections and significance, see legend to Fig. 1.

Significance: Soluble protein: E_3 vs E_4: NS; E_3 vs TAM: $P<0.01$.

due to the second dose, since the rise between 24–36 h after a single injection was comparable to that at 48 h after the first of 2 injections. In contrast to the gain in wet weight and luminal fluid content after the third injection of E_2, only small changes were observed in DNA and total protein (Figs. 3C,D).

Morphologic Findings

Treatment with all four compounds resulted in quantitative and qualitative morphologic changes indicative of enhanced synthetic and secretory activity, as evaluated by light and electron microscopy. Luminal epithelial cells changed from cuboidal to tall columnar after E_3 or E_4 injections. Glandular epithelial cells attained a tall columnar shape only after TAM treatment (Fig. 4).

Ultrastructural studies (Figs. 5a–d) showed that treatment with E_2 or TAM promoted the development of prominent Golgi systems and rough endoplasmic reticulum in luminal epithelial cells. Secretory granules containing a dense core and a lucent halo were seen in their apices (Figs. 5b,d). Similar but less pronounced changes were observed after E_3 or E_4 treatment but secretory granules were absent. Microvilli were largest after E_2 or TAM treatment, but were more abundant only after E_2. Degeneration was observed by light microscopy (Figs. 4b–d) and confirmed by electron microscopy (Fig. 4f). Degeneration occurred in luminal and glandular epithelium after E_3 or E_4 and to a smaller degree after TAM treatment. Estradiol produced this effect in glandular but not in luminal epithelium. Ultrastructural changes in the glandular epithelial cells generally paralleled those in luminal epithelium. No nuclear bodies were observed in epithelial cells either in controls or in any of the treatment groups. In contrast, considerable numbers of nuclear bodies have been found in the immature rat uterus at an earlier age (LeGoascogne and Baulieu, 1977).

Table 2 shows that the accumulation of eosinophils throughout the uterus was strongly promoted by E_2, an effect observed to a lesser, though statistically significant extent also after E_3 or E_4, but not after TAM injection. Mast cells were significantly decreased after E_3, E_4 or TAM, but not after E_2 treatment.

The ratios of the endometrial/myometrial cross-sectional areas decreased after E_2, E_3 or E_4, but increased significantly after TAM treatment. This indicates that TAM, in contrast to the other compounds, acted preferentially on the endometrium.

Table 3 summarizes, for purposes of comparison, the various biochemical and morphologic changes produced by E_2, E_3, E_4 and TAM.

DISCUSSION

The results of this study demonstrate an estrogenic response of the immature rat uterus to E_4 and TAM, as well as to E_2 and E_3, which were measured for comparison. At the doses and injection schedule chosen for these experiments, this response occurred in a gradient in most biochemical, histological and ultrastructural parameters. With few exceptions, E_2 treatment promoted the most marked changes, followed by TAM, E_3 and E_4.

The total uterine progesterone receptor (PR) content as well as PR per cell rose significantly after treatment with all four compounds. However, when PR was expressed per mg cytosol protein, the increase was significant only after E_2 and TAM treatment; it was small after E_3 and negligible after E_4 injection. This suggests that after E_3 and E_4 treatment the increases in total uterine PR or PR per cell occurred as part of a general augmentation in protein synthesis which followed all treatments,

TABLE 1. Biochemical responses of the immature rat uterus to treatment for 3 days with estrogens or tamoxifen.

Parameter	Series[a]	Treatment: Oil	E_2	E_3	E_4	Tamoxifen
Uterine wet weight[b] (mg)	A	33.2 ± 1.9[c] (15)	131.3 ± 5.3[d]*** (10)	62.4 ± 2.3*** (15)	50.1 ± 1.9*** (15)	73.9 ± 2.7*** (15)
Progesterone receptor/ total protein (pmol/mg)	B/A	0.18	0.39	0.29	0.23	0.31
Progesterone receptor content (pmol/uterus)	B	0.63 ± 0.03 (8)	5.83 ± 0.30*** (6)	1.78 ± 0.10*** (8)	1.28 ± 0.10***(8)	2.51 ± 0.15*** (7)
DNA content (mg/uterus)	A	0.39 ± 0.03	0.75 ± 0.04***	0.53 ± 0.03**	0.45 ± 0.04	0.50 ± 0.07
	B	0.37 ± 0.08	0.67 ± 0.02***	0.52 ± 0.01***	0.46 ± 0.05***	0.50 ± 0.03***
DNA concentration (mg/100 mg tissue)	A	1.17 ± 0.07	0.57 ± 0.02***	0.85 ± 0.04***	0.90 ± 0.07*	0.65 ± 0.07***
	B	1.23 ± 0.02	0.51 ± 0.02***	0.82 ± 0.03***	0.96 ± 0.03***	0.70 ± 0.02***
Total protein content (mg/uterus)	A	3.48 ± 0.25	15.00 ± 0.48***	6.27 ± 0.25***	5.71 ± 0.26***	8.23 ± 0.30***
Total protein concentration (mg/100 mg tissue)	A	10.40 ± 0.29	11.49 ± 0.32*	10.09 ± 0.27	11.45 ± 0.39*	11.18 ± 0.24*
Cytosol protein content (mg/uterus)	B	0.99 ± 0.04	6.48 ± 0.32***	2.41 ± 0.12***	1.95 ± 0.17***	2.63 ± 0.13***
Cytosol protein concentration (mg/100 mg tissue)	B	3.28 ± 0.09	4.83 ± 0.15***	3.75 ± 0.12**	3.99 ± 0.18**	3.71 ± 0.14*
Luminal fluid content (mg/uterus)	A	1.8 ± 0.2	76.5 ± 10.0***	2.6 ± 0.2**	1.9 ± 0.1	3.1 ± 0.2***

[a] Experimental series A and B. For details, see Materials and Methods.

[b] Weights after removal of luminal fluid.

[c] Mean ± SEM. The n rats in series A and B are given in parentheses.

[d] Significance by Student's two-tailed t test. Significantly different from control at ***$P<0.001$, **$P<0.01$, *$P<0.05$.

E_2 differed significantly from E_3 in the following parameters: uterine weight ($P<0.001$); progesterone receptor content ($P<0.01$); DNA content ($P<0.05$) and concentration ($P<0.01$), series B; cytosol protein content ($P<0.05$); total protein concentration ($P<0.01$); luminal fluid ($P<0.01$).

Tamoxifen differed significantly from E_2 in: uterine weight ($P<0.001$); progesterone receptor content ($P<0.01$); DNA concentration, series A and B ($P<0.05$); total protein content ($P<0.001$) and concentration ($P<0.01$); luminal fluid ($P<0.05$).

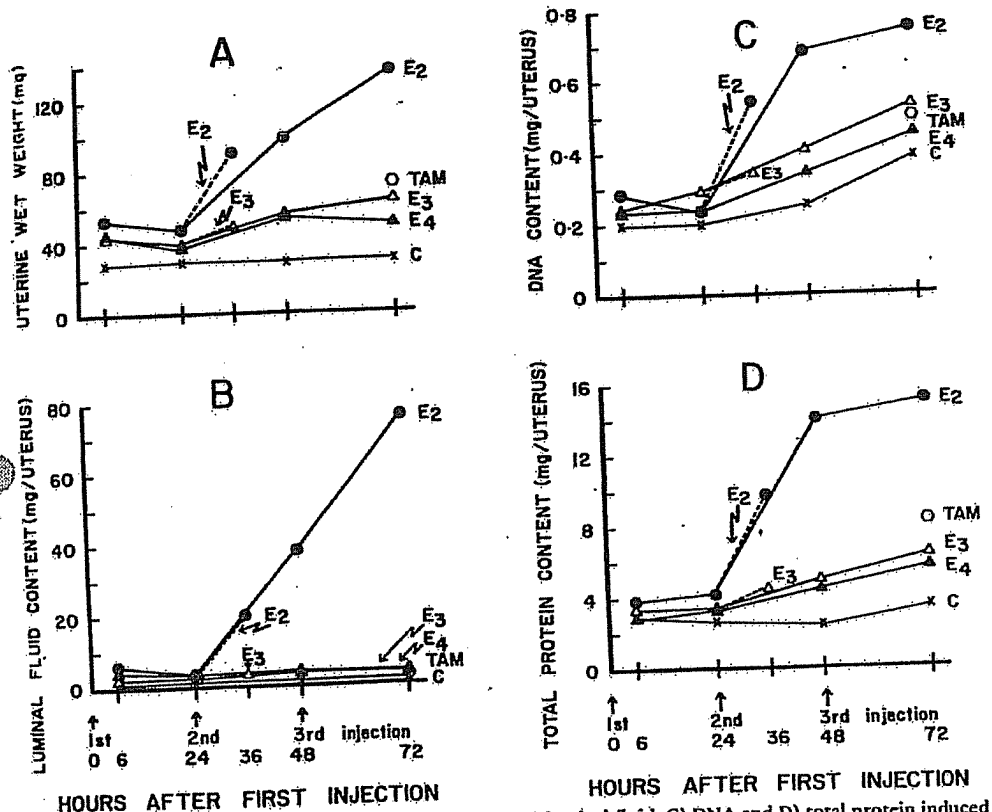


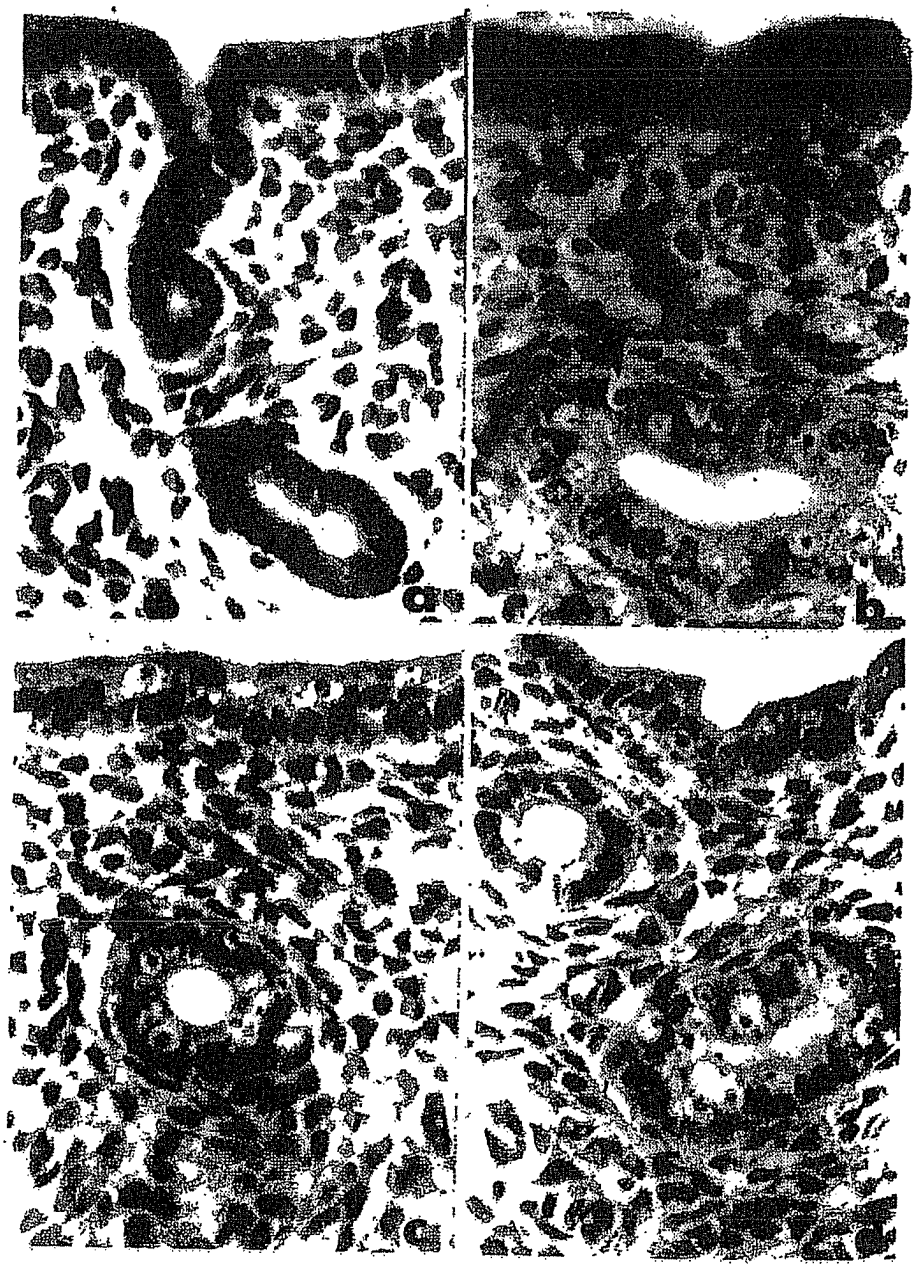
FIG. 3. Time course of changes in A) uterine weight, B) luminal fluid, C) DNA and D) total protein induced by treatment with E_2, E_3 or E_4. Injections were given at 24 h intervals, starting at 0 h. Values at 24, 48 and 72 h were obtained after 1, 2 or 3 injections, respectively. Effects of E_2 and E_3 at 36 h were measured after a single injection at 0 h. Changes at 72 h after 3 daily injections of TAM are given for comparative purposes. For dosages, see legend to Fig. 1. A part of the above data, used here for comparison, was previously reported (Holinka and Gurpide, 1979).

as indicated by the significant rise in soluble and total protein per DNA.

However, this interpretation merits caution because it is based on changes of two parameters (PR and cytosol protein) in three cell types (epithelial, stromal and myometrial). Both parameters may vary independently and that variation may occur to a different extent in the three cell populations, as was recently shown in relation to estrogen receptor changes in the immature rat uterus in response to short term E_2 treatment (McCormack and Glasser, 1978). It is possible that the significant increases in total PR after E_4 injections observed here reflect specific PR synthesis in the endometrium, while the rise in soluble (cytosol) protein occurred mainly in the myometrium.

Whether by enhanced general protein synthesis or specific receptor induction, E_4 and TAM increased the total PR population per cell; since target tissue responsiveness to a steroid hormone is in part reflected by the presence of cytoplasmic receptors for that hormone, it can be concluded that E_4 and TAM are capable of enhancing uterine responsiveness to progesterone. The ability of TAM to augment the PR concentration has previously been observed in the rodent uterus (Koseki et al., 1977b; Jordan et al., 1978; Bichon and Bayard, 1979), as well as in human endometrium (Robel et al., 1978). On the basis of these observations, it has been suggested that TAM might be a compound of choice in enhancing the therapeutic effect of progesterone with a minimum of estrogenic side effects (Robel et al., 1978).

The histologic changes in luminal epithelium induced by TAM appeared similar to those observed in immature rat uteri after nafoxidine treatment (Clark et al., 1978). Both triphenylethylene derivatives caused a marked increase in the height of the luminal epithelium which, in the present study, exceeded that induced by E_2. Electron microscopy revealed TAM-induced morphologic changes suggestive of increased protein synthesis and enhanced secretory and



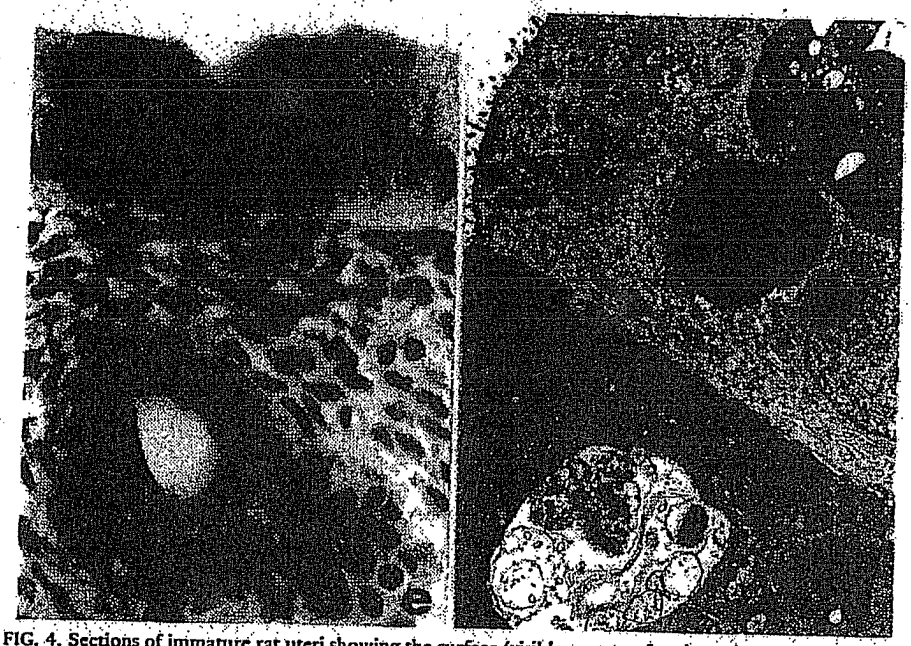


FIG. 4. Sections of immature rat uteri showing the surface (visible on top of each section, a–e) and glandular epithelium: a) control: cuboidal to low columnar epithelium with nuclei occupying most of the cell volume; b) E_2 treated: tall columnar epithelium with numerous degenerating cells in the glands (arrowhead) but not in the luminal surface; c) E_3- and d) E_4-treated: low columnar epithelium with degenerating cells in both surface and glandular epithelium; e) tamoxifen-treated: tall columnar epithelium with little indication of degeneration; f) electron micrograph of part of gland in an E_2-treated rat demonstrating a lysosome with substantially degenerated contents corresponding to the pale areas in b–d. Lysosomes exhibiting less degraded contents are seen in the upper right corner. a–e, X480; f, X4,900.

absorptive activity, such as prominent rough endoplasmic reticulum (RER), enlarged Golgi systems and an elongation of the microvilli. The ultrastructural changes in glandular epithelium, i.e., an abundance of secretory granules and prominent RER, likewise indicate augmented secretory activity.

In agreement with previous reports on the immature rat uterus (Jordan, 1976; Jordan et al., 1978), TAM promoted increases in uterine wet weight and DNA. In addition, the present data show increased cytosol and total protein levels and a preferential effect on the endometrium.

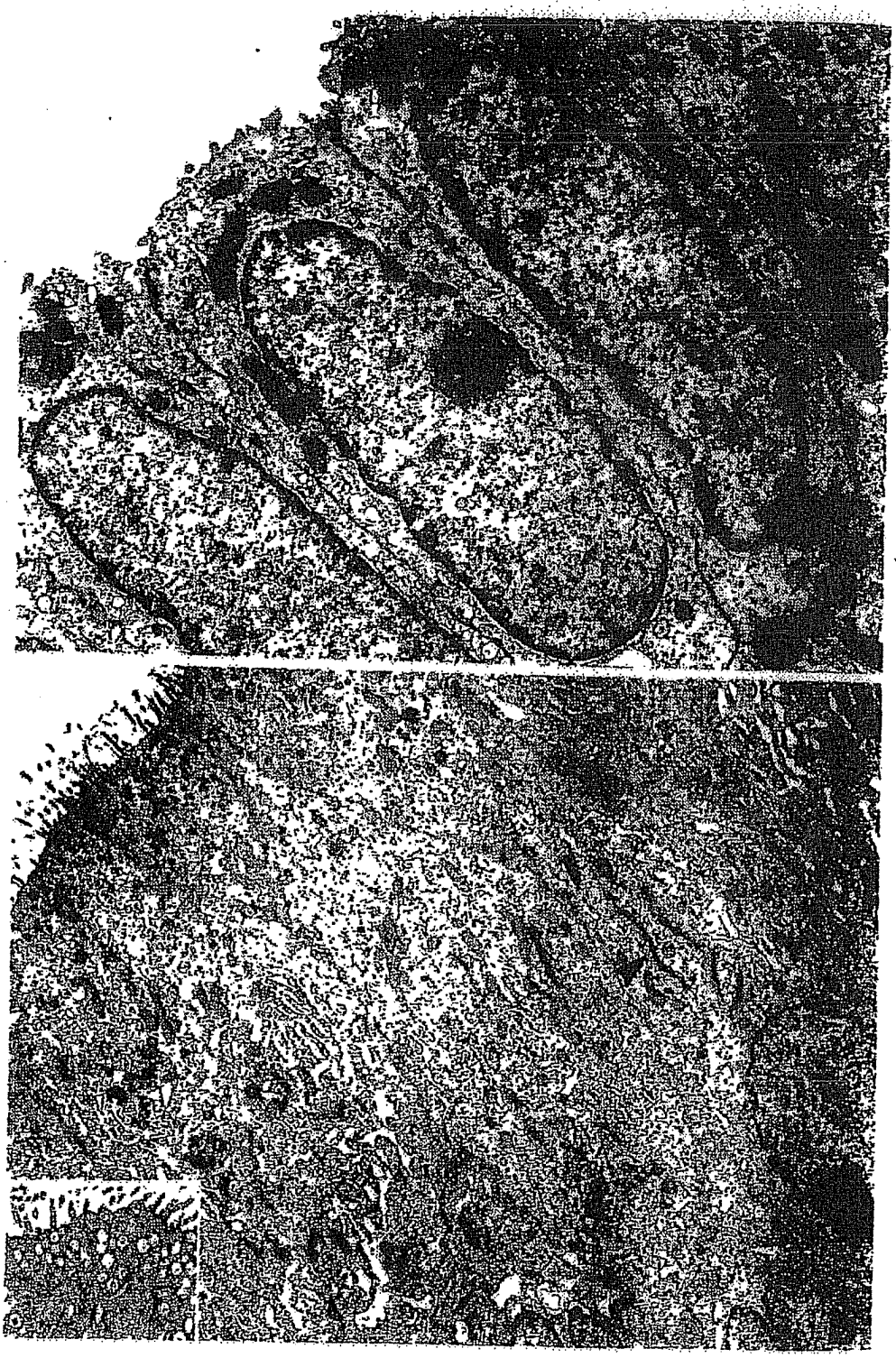
Estradiol and E_3 were found equally effective in short term induction of eosinophilia, measured 6 h after a single injection (Tchernitchin et al., 1976a,b). In contrast, our data show that E_3 was not able to sustain this effect on a long term basis.

FIG. 5. a) Electron micrograph of the luminal surface epithelium of a control rat uterus. Microvilli are short and sparse. The cytoplasm is scant and exhibits relatively few organelles. X10,400.

b) Luminal surface epithelium of an E_2-treated rat. The rough endoplasmic reticulum is abundant and contains many dilated profiles. Golgi systems are extensive (arrowheads). Secretory granules are present just beneath the elongated microvilli. Inset: illustration of secretory granules containing a dense core with a clear halo. X5,000; inset, X6,000.

c) Luminal surface epithelium of an E_4-treated rat. Organelles of secretion and microvilli are more abundant than in the control but less prominent than in the E_2-treated rats. Nuclei are highly irregular in outline. X10,400.

d) Luminal surface epithelium of a tamoxifen-treated rat. As with E_2-treatment, organelles involved in secretion are abundant and secretory granules are present (arrowhead). X10,400.



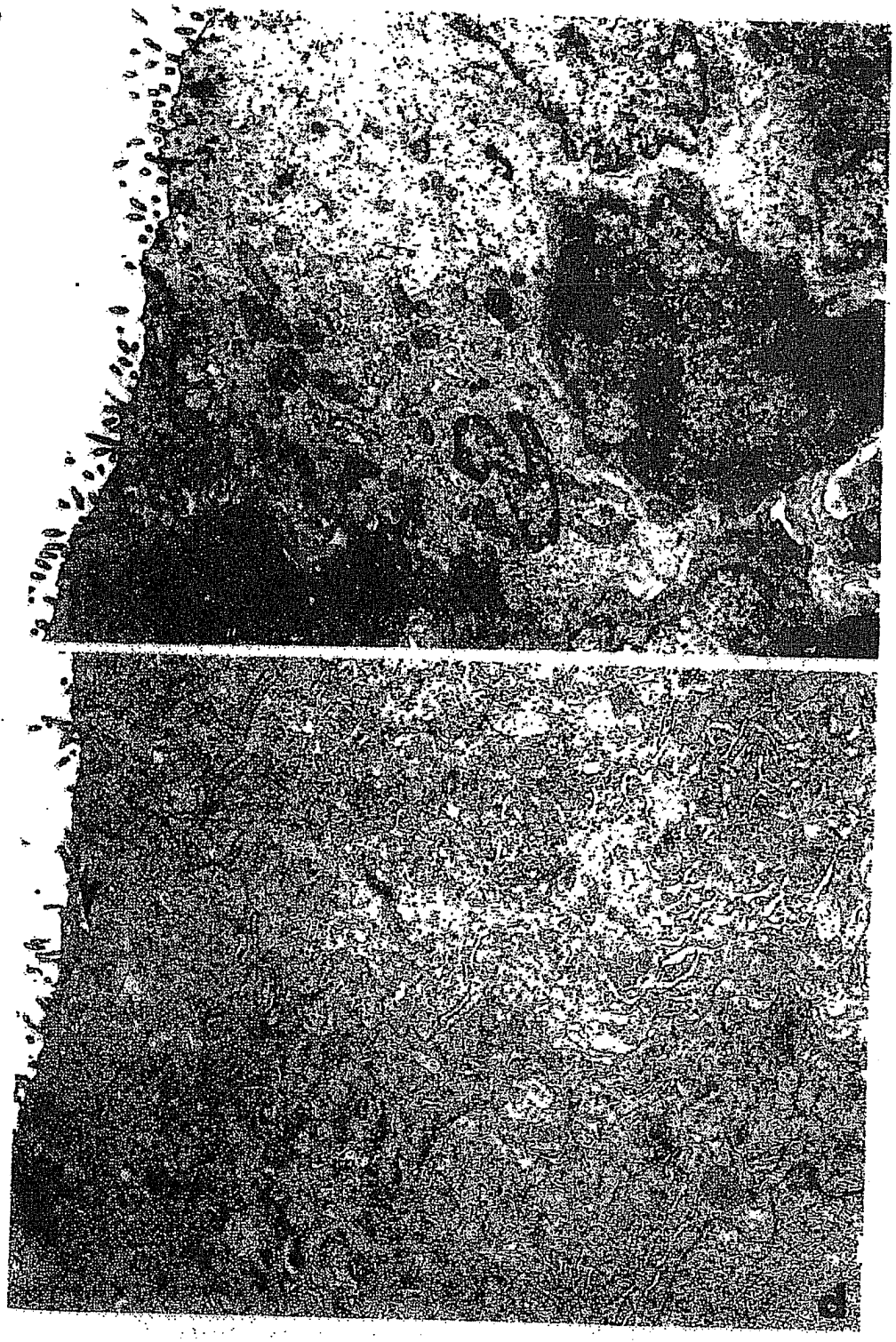


TABLE 2. Changes in ratios of endometrial/myometrial cross-sectional areas and in numbers of eosinophils and mast cells in the uterus of immature rats injected with estrogens or tamoxifen for 3 days.

Parameter	Treatment				
	Oil	E_2	E_3	E_4	Tamoxifen
Ratio: endometrium/myometrium	0.86 ± 0.08	0.73 ± 0.11	0.73 ± 0.07	0.67 ± 0.03[a]	1.36 ± 0.04***
Eosinophils/cross section	0.2 ± 0.1	130.4 ± 17.5***	4.2 ± 1.1**	4.5 ± 0.9***	0.3 ± 0.1
Mast cells/cross section	8.6 ± 1.4	5.3 ± 1.0	1.2 ± 0.5***	2.1 ± 0.3***	2.2 ± 0.4***

[a] For key to significance levels, see Table 1. The differences between E_3 and E_4 were not significant. Tamoxifen differed significantly from E_2 in endometrial/myometrial ratios ($P<0.001$) and eosinophils ($P<0.01$).

The slight but steady rise in control values of DNA, but not of wet weight or total protein, between 24–72 h may indicate some mitotic activity without hypertrophy as a part of uterine development in 25–27-day-old rats.

All four compounds promoted highly significant increases in soluble (cytosol) protein as well as total protein per cell. It appears, however, that E_4 and TAM were not able to influence soluble protein synthesis selectively, since only E_2 and E_3 treatment produced a marked increase in soluble/total protein ratios over control values. Since most myosin and much of the thin filament protein of smooth muscle are extractable at low ionic strength (Murphy and Megerman, 1977), it is possible that increased protein levels of myometrial origin contributed to the elevation in soluble/total protein ratios in response to E_2 and E_3 treatment. This effect was not obtained by TAM administration, which appears to have stimulated only the endometrium.

Results of other investigators (Anderson et al., 1975; Harris and Gorski, 1978) have shown that the effects of E_3 on uteri of sexually immature rodents are influenced not only by the dose administered but also by the schedule of administration. This consideration is relevant to the evaluation of the estrogenicity of E_4 in the present series of experiments, since it is possible that a continuous supply of E_4 may lead to stronger estrogenic effects. It should be mentioned in this connection that E_4 increases the levels of progesterone receptors and the incorporation of [^{3}H]-thymidine in MCF-7 cells when added to the culture medium (Kreitmann and Bayard, 1979).

In spite of this limitation, it can be concluded that E_4 is capable of stimulating uterine growth and protein synthesis, although it is considerably less effective than E_3 in promoting an increase in uterine DNA under the same injection schedule. The present results, which contrast with those from other studies concluding that E_4 had no effects on the immature rat uterus (Fishman and Martucci, 1978), are pertinent to questions related to the role that the compound formed endogenously during human pregnancy plays in the development of the fetus.

ACKNOWLEDGMENTS

Miss Elizabeth Storch provided skillful technical assistance in the ultrastructural studies.

TABLE 3. Summary of relative uterine changes in biochemical and morphologic parameters after estrogen or tamoxifen treatment for 3 days.

Parameter increases	Compound (dose/100 g BW)			
	E_2 (1 μg)	E_3 (1 μg)	E_4 (50 μg)	Tamoxifen (50 μg)
Biochemical				
Uterine wet weight	+++++			+++
Luminal fluid weight	+++++	++	+	+
DNA/uterus	+++	+	−	±
Total protein/DNA	++++	+	±	±
Soluble protein/total protein	++	+	+	++++
P receptor/DNA	+++++	++	−	−
P receptor/soluble protein	++	±	+	+++
Morphologic			−	++
Luminal epithelium				
Effect on height	+++	++	++	++++
Microvilli number	++++	++	++	++++
Microvilli length	++++	++	++	++++
Golgi systems	++++	++	++	++++
RER	++++	++	++	++++
Cell degeneration	−	++++		±
Glandular epithelium			+	++++
Effect on height	++	+	±	++++
RER	++++	±	−	++++
Secretory granules	++++	−	++++	++++
Cell degeneration	++++	++++	−[a]	±
Endometrium/myometrium ratio	−[a]	+	+	++++
Eosinophil number	++++	++	++	−
Mast cell decrease	−			++

[a] A marked and comparable decrease was observed in these 3 treatments.

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EXHIBIT "F"

Clinical Gynecologic Endocrinology and Infertility

Seventh Edition

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dehydrogenase and the mechanism appeared to be independent of the estrogen receptor.[66] The increase in DHAS secretion by the fetal zone is a consequence of suppression of the gene *(HSD3B2)* that controls 3β-hydroxysteroid dehydrogenase expression; transcriptional factors necessary for the activity of this gene are absent in the fetal zone.[67] The hyperplasia of the fetal adrenal may be the result of the high ACTH levels due to the relatively low cortisol levels, a consequence of the enzyme inhibition.

The development of the adrenal gland during human fetal life and during the neonatal period is paralleled in the baboon.[68] The adrenal cortex of the fetal baboon is characterized by the same deficiency in 3β-hydroxysteroid dehydrogenase as that seen in the human, with the same diversion of steroidogenesis into production of DHAS. Treatment of the neonatal baboon with estrogens and progesterone did not halt the regression of the fetal zone and DHAS production, arguing against the hypothesis that the fetal zone is dependent on an estrogen-induced deficiency in 3β-hydroxysteroid dehydrogenase. Treatment of the pregnant baboon with estradiol reduced production of DHAS.[69] It continues to be uncertain, however, whether the internal microenvironment of the adrenal gland can be affected by the exogenous administration of steroids. In the monkey, epidermal growth factor can increase the 3β-hydroxysteroid dehydrogenase content in the fetal adrenal gland, but it is not clear how this action is regulated.[70]

This explanation (estrogen regulation of 3β-hydroxysteroid dehydrogenase) is further challenged by in vitro studies of human fetal zone cells indicating that estradiol and IGF-II combine to direct steroidogenesis to DHAS in a mechanism not due to inhibition of 3β-hydroxysteroid dehydrogenase.[63]

Nevertheless, it is an attractive and useful hypothesis to view the principal mission of the fetal adrenal as providing DHAS as the basic precursor for placental estrogen production. Estrogen, in turn, feeds back to the adrenal to direct steroidogenesis along the Δ^5 pathway to provide even more of its precursor, DHAS. Thus far, this is the only known function for DHAS. With birth and loss of exposure to estrogen, the fetal adrenal gland quickly changes to the adult type of gland.

Measurement of Estrogen in Pregnancy

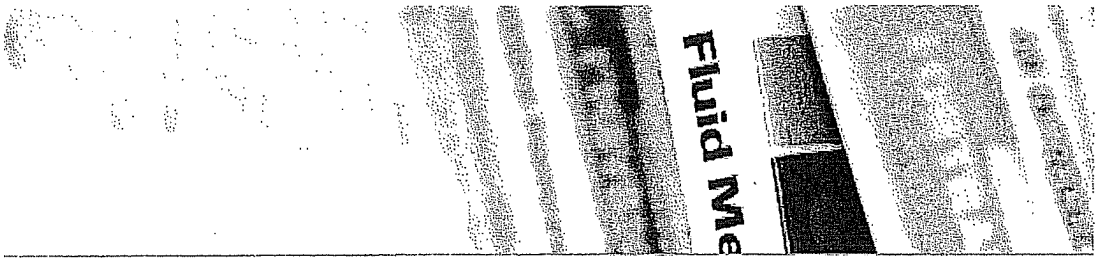
Because pregnancy is characterized by a great increase in maternal estrogen levels and estrogen production is dependent on fetal and placental steroidogenic cooperation, the amount of estrogen present in the maternal blood or urine reflects both fetal and placental enzymatic capability and, hence, well-being. Attention focused on estriol because 90% of maternal estriol is derived from fetal precursors. The end product to be assayed in the maternal blood or urine is influenced by a multitude of factors. Availability of precursor from the fetal adrenal gland is a prime requisite, as well as the ability of the placenta to perform its conversion steps. Maternal metabolism of the product and the efficiency of maternal renal excretion of the product can modify the daily amount of estrogen in the urine. Blood flow to any of the key organs in the fetus, placenta, and mother becomes important.[71, 72] Fetal hypoxemia due to reduced uteroplacental blood flow is associated with a marked increase in adrenal androgen production in response to an increase in fetal ACTH and, in response to the availability of androgen precursors, an increase in maternal estrogen levels.[73] The response to acute stress is in contrast to the effect of chronic uteroplacental insufficiency, which is associated with a reduction in fetal androgens and maternal estrogens. In addition, drugs or diseases can affect any level in the cascade of events leading up to the assay of estrogen.

For years, measurement of estrogen in a 24-hour urine collection was the standard hormonal method of assessing fetal well-being. This was replaced by immunoassay of unconjugated estriol in the plasma.[74] Because of its short half-life (5–10 minutes) in the maternal circulation, unconjugated estriol has less variation than urinary or total blood estriol. However, assessment of maternal estriol levels has been superseded by various biophysical fetal monitoring techniques such as nonstress testing, stress testing, and measurement of fetal breathing and activity. Modern screening for fetal aneuploidy (discussed later in the chapter) utilizes three markers in the maternal circulation: alpha fetoprotein, human chorionic gonadotropin, and unconjugated estriol.

Amniotic Fluid Estrogen Measurements

Amniotic fluid estriol is correlated with the fetal estrogen pattern rather than the maternal. Most of the estriol in the amniotic fluid is present as 16-glucosiduronate or as 3-sulfate-16-glucosiduronate. A small

EXHIBIT "G"



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The pharmacokinetics of intravenous estradiol: A preliminary study

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Résumé / Abstract
Estradiol USP was extemporaneously compounded for intravenous administration. Eight postmenopausal women were randomized to receive one of four estradiol dosages. Serum estradiol concentrations were determined at frequent intervals after single bolus dosing. The concentration-time profile was stripped and fit, and pharmacokinetic values were generated. Approximate dosage proportionality was seen with area under the curve, the terminal half-life was 27.45 ± 5.65 minutes, and volume of distribution was very low (0.082 ± 0.015 L/kg). Estradiol was well tolerated by all study participants.

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Estetrol does not bind sex hormone binding globulin or increase its production by human HepG2 cells

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Key words: ESTETROL, E_4, PLASMA, SEX HORMONE BINDING GLOBULIN

ABSTRACT

Objectives To determine whether human sex hormone binding globulin (SHBG) binds estetrol (E_4), and to assess whether E_4 stimulates the production of SHBG by human hepatocytes.

Methods Competitive ligand binding assays have been used to assess the relative binding affinity of E_4 to human SHBG using either [^{3}H]5α-dihydrotestosterone or [^{3}H]estradiol as labeled ligands. The effect of E_4 on the production of SHBG has been assessed by a fluoroimmunometric assay in wild-type human HepG2 cells and in human Hep89 cells that over-express the human estrogen receptor (ER) α, and compared to the effect of ethinylestradiol, estradiol and estriol.

Results There was no detectable binding of E_4 to the human SHBG steroid-binding sites. By contrast, testosterone and estradiol were bound with high affinity and the synthetic estrogen ethinylestradiol was found to bind SHBG with low affinity. Estetrol does not stimulate ERα-mediated increases in SHBG production by HepG2 or Hep89 cells, in contrast to ethinylestradiol, estradiol and estriol.

Conclusions These data indicate that SHBG has no influence on the plasma distribution of E_4 or its availability to target tissues. In addition, it is shown that E_4 has no effect on SHBG production by human hepatocytes.

INTRODUCTION

The liver is the major site of synthesis of plasma steroid-binding proteins, including sex hormone binding globulin (SHBG). These plasma steroid-binding proteins not only transport steroids in the blood but, in some cases, function to regulate their access to target tissues[1]. Most natural androgens and estrogens, and several synthetic sex steroids, bind to human SHBG with high affinity, and these interactions determine the distribution of these steroids between the protein-bound and non-protein-bound fractions in blood plasma. This is important because only non-protein-bound or 'free' steroids are thought to gain access to target tissues[2]. Increases in the plasma concentrations of

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SHBG occur after administration of exogenous estrogens and during pregnancy due to the increase of endogenous estrogens. This increase in SHBG can have a marked impact on the plasma distribution and tissue availability of endogenous as well as exogenous SHBG ligands. For instance, oral administration of ethinylestradiol (EE) as a contraceptive agent will increase plasma SHBG levels 5–10-fold. This not only reduces 'free' testosterone levels but will also reduce the 'free' levels of several contraceptive progestins that bind with high affinity to human SHBG, and which are often co-administered in the drug formulation.

In the development of any new estrogenic pharmaceutical, it is therefore important to evaluate its effects on plasma SHBG levels and to determine whether binding to SHBG will influence its access to target tissues. In this report, the relative binding affinity (RBA) of human SHBG for estetrol (E_4) has been evaluated and compared with natural androgens and the natural estrogen estradiol (E_2), as well as the widely used synthetic estrogen, EE, which is known to bind human SHBG with low affinity[3,4]. This was accomplished using an established competitive steroid-binding assay[5]. In these assays, both radiolabeled androgen and E_2 were used as tracer because recent crystallography experiments have indicated that androgens and estrogens occupy the human SHBG steroid-binding site in quite different orientations[6].

This study has also investigated whether administration of E_4 influences the hepatic production and secretion of SHBG in a hepatocyte cell model, which consequently may predict changes in its plasma levels. This was done by culturing, in the absence and presence of E_4, the human hepatoma cell line HepG2 and the hepatoma cell line Hep89, over-expressing the estrogen receptor (ER) α. Other estrogens were used as control, including E_2, the weak natural estrogen, estriol (E_3), and the potent synthetic estrogen, EE.

METHODS

Steroids

Estradiol, E_3, EE, testosterone and 5α-dihydrotestosterone (DHT) were purchased from Steraloids Inc. (Wilton, NH, USA) and used without further purification. Estetrol was supplied by Syncom (Groningen, The Netherlands) and its purity was validated by the supplier. Stock solutions of unlabeled steroids were prepared in ethanol and diluted further in phosphate buffered saline (PBS) or culture medium, as indicated below. Radiolabeled steroids [^{3}H]DHT and [^{3}H]E_2 were obtained from PerkinElmer Life Sciences (Boston, MA, USA) and used without further purification.

Human SHBG

Human SHBG was purified from transgenic mouse serum, as described previously[7]. The human SHBG prepared in this way was assessed to be >99% pure by polyacrylamide gel electrophoresis under denaturing conditions, and its steroid-binding characteristics are indistinguishable from SHBG purified from human serum[7].

SHBG steroid binding assays

An established competitive steroid-binding assay was used to determine the relative binding affinity of E_4 for human SHBG[5]. This assay involves the use of pure human SHBG (see above) and [^{3}H]DHT or [^{3}H]E_2 as labeled ligands. In brief, human SHBG was treated for 30 min at room temperature with a dextran-coated charcoal (DCC) suspension in phosphate buffered saline (PBS) to remove steroid ligand. After centrifugation (2000 × g for 10 min) to sediment the DCC, the supernatant containing the human SHBG was diluted in PBS to a concentration of 1 nmol/l based on its steroid binding capacity. Duplicate aliquots (100 μl) of this human SHBG solution were then incubated with an equal volume of either [^{3}H]DHT or [^{3}H]E_2 at 10 nmol/l, together with 100 μl of PBS alone or the same amount of PBS containing increasing concentrations of unlabeled steroid ligands as competitors in polystyrene test tubes. After incubation for 1 h at room temperature, the reaction mixtures were placed in an ice bath for a further 15 min. Aliquots (600 μl) of an ice-cold suspension of DCC were then added to each tube and, after a brief 2 s mixing, each tube was incubated in an ice bath for either 10 min or 5 min, depending on whether [^{3}H]DHT or [^{3}H]E_2 was being used as labeled ligand, respectively. The unbound ligands adsorbed to DCC were then removed by centrifugation (2000 × g for 15 min at 4°C), and the amounts of ^{3}H-labeled ligands bound to SHBG bound in the supernatants were counted in 2 ml scintillation cocktail using a liquid scintillation spectrophotometer (PerkinElmer Life Sciences, Turku, Finland).

The average amounts of ^{3}H-labeled ligands bound to SHBG at each concentration of competitor were expressed as a percentage of the average

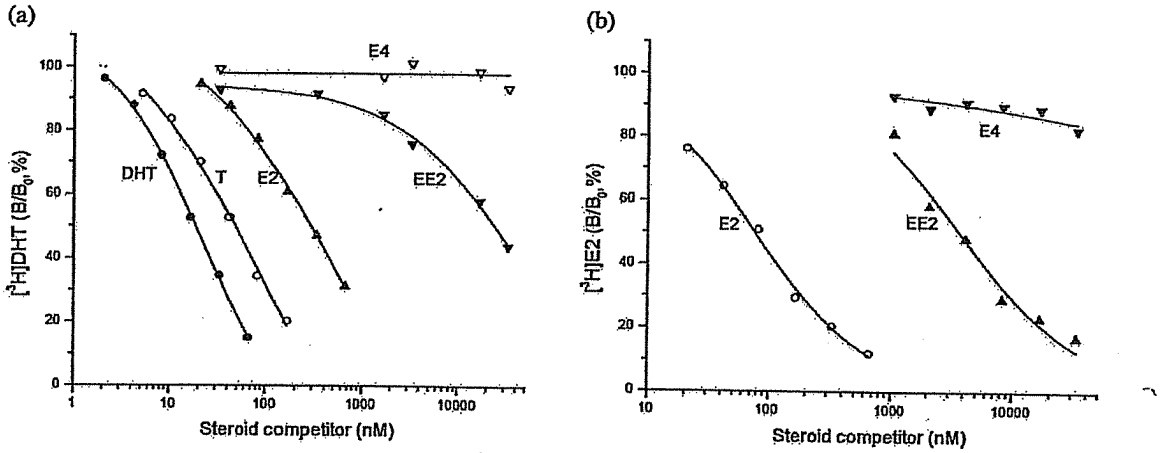

Figure 1 Competitive displacement of [^{3}H]DHT (a) and [^{3}H]E2 (b) from the human sex hormone binding globulin steroid-binding site. The unlabeled steroid ligands used as competitors were as follows: DHT, 5α-dihydrotestosterone; T, testosterone; E2, estradiol; EE2, ethinylestradiol; E4, estetrol

amounts of ^{3}H-labeled ligands bound to SHBG in the absence of competitor, and were plotted against the concentration of competitor in each assay tube. The RBA for each competitor was then determined as a ratio of the concentration of steroid competitor resulting in a 50% reduction in specific binding of [^{3}H]DHT or [^{3}H]E_2 relative to the concentration of DHT or E_2 required to produce the same effect, respectively.

SHBG production assays

Wild-type HepG2 cells from the American Tissue Type Collection and Hep89 cells that over-express the human ERα (provided by Dr Michel Pugeat) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing phenol red and 10% fetal bovine serum (FBS) in the presence of antibiotics (penicillin and streptomycin). Prior to the experiments, cells were passaged once in phenol-red free DMEM + 5% charcoal-stripped FBS and antibiotics. They were then plated at 50 000 cells/well in 24 well plates and grown to 80–90% confluence. The medium was removed and the cells were washed with PBS prior to the addition of phenol-red free culture medium that either lacked (control) or contained increasing concentrations (0.1 nmol/l–1 μmol/l) of E_2, E_3, E_4 or EE. Aliquots (0.25 ml) of medium were removed from the cell cultures in 24 well plates at 24, 48 and 72 h thereafter, for measurements of human SHBG concentrations using a sensitive and specific time-resolved fluoroimmunometric assay[8].

Table 1 Relative binding affinities of steroid ligands for human SHBG compared to DHT or estradiol

Ligand tested	*Relative binding affinity (RBA)*
*DHT competition assay**	
DHT	100.00
Testosterone	40.00
Estradiol	6.80
Ethinylestradiol	0.08
Estetrol	≫0.00
*Estradiol competition assay***	
Estradiol	100.00
Ethinylestradiol	2.02
Estetrol	≫0.00

*RBA determined as a ratio of the concentration of steroid competitor resulting in a 50% reduction in specific binding of [^{3}H]DHT to the concentration of DHT required to produce the same effect multiplied by 100

**RBA determined as a ratio of the concentration of steroid competitor resulting in a 50% reduction in specific binding of [^{3}H]E2 to the concentration of estradiol required to produce the same effect multiplied by 100

RESULTS

Estetrol does not bind to human SHBG

The results of the competitive steroid binding assays using pure human SHBG together with [^{3}H]DHT and [^{3}H]E_2 are shown graphically in panels (a) and (b), respectively, of Figure 1. These data show unequivocally that E_4 does not interact at all with the human SHBG steroid-binding site.

By comparison, the natural steroid ligands E_2 and testosterone of human SHBG produced the expected displacement curves, and displacement of both [^{3}H]DHT and [^{3}H]E_2 from the SHBG steroid-binding site was also observed to a lesser extent with the synthetic estrogen EE. A summary of the RBAs of ligands tested in these assays is also presented in Table 1.

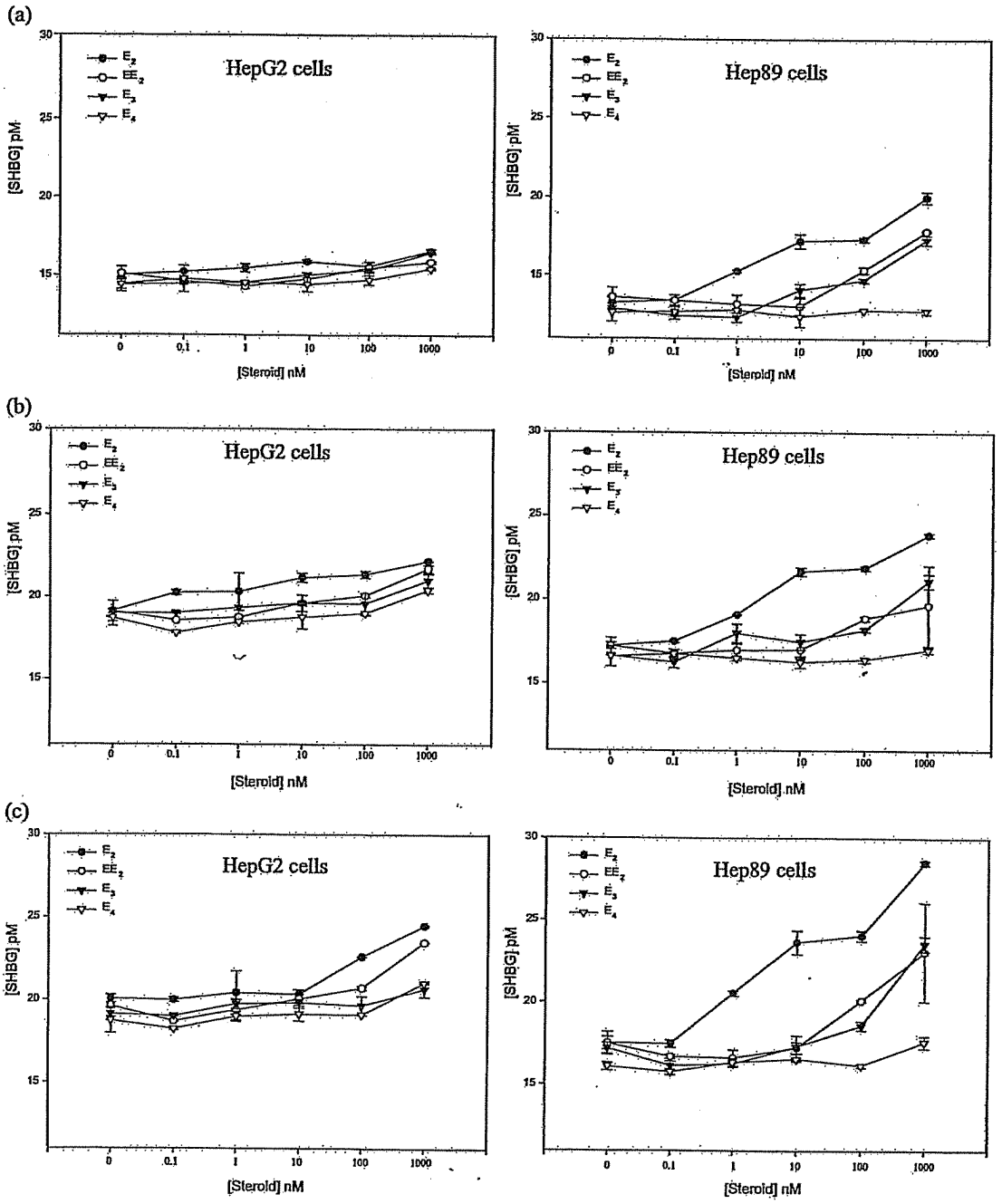

Figure 2 Human SHBG concentrations (pmol/l) in 100 µl culture medium of HepG2 and Hep89 cells after (a) 24, (b) 48 and (c) 72 h exposure to increasing concentrations of estetrol (hollow trianges, E4), estriol (solid trianges, E3), ethinylestradiol (hollow circles, EE2) and estradiol (solid circles, E2). Data points represent means ± SEM of duplicate measurements of samples taken from duplicated cultures

Estetrol does not stimulate ERα-mediated increases in SHBG production by HepG2 or Hep89 cells

When wild-type HepG2 cells were exposed to phenol-red free medium containing 0.1 nmol/l–1 μmol/l concentrations of estrogens, E_3 or E_4 failed to increase the SHBG accumulation in the medium even after 72 h, and significant increases in SHBG accumulation in the medium were only observed at 0.1 and 1 μmol/l E_2 and 1 μmol/l EE at this time point (Figure 2). The estrogenic response in Hep89 cells was clearly more robust with respect to the increased SHBG accumulation in the medium, which was already evident within 24 h of treatment with as little as 1 nmol/l E_2 (Figure 2). Increased SHBG accumulation was also observed after 24 h of treatment with 100 nmol/l EE or with 1 μmol/l E_3 (Figure 2). By contrast, E_4 failed to increase SHBG production by the Hep89 cells in a dose-dependent manner over the 72-h incubation period (Figure 2).

DISCUSSION

Estrogens are known to interact with SHBG metabolism by (1) increasing its production and (2) binding to it. Estetrol has not been studied as a potential human SHBG ligand previously.

The binding of steroid ligands to human SHBG was assessed using both [^{3}H]DHT and [^{3}H]E_2 as labeled ligands. The RBA data obtained using [^{3}H]DHT as labeled ligand are in good agreement with previous reports[1]. The results clearly indicate that E_4 is incapable of displacing [^{3}H]DHT from the human SHBG steroid-binding site, even at concentrations approaching its solubility limit in the assay buffer. This was also confirmed by using [^{3}H]E_2 as labeled ligand. In these studies, EE was used as an example of a low affinity SHBG ligand. In the assay in which [^{3}H]DHT was used as labeled ligand, EE displayed an approximately 90-fold lower affinity for human SHBG when compared to E_2. This is in line with several previous estimates of the RBA of human SHBG for EE using different assay methodologies[3,4]. When [^{3}H]E_2 was used as the labeled ligand, EE displayed an approximately 50-fold lower affinity for human SHBG when compared to E_2. This can be explained by the different orientation of androgens and estrogens in the human SHBG steroid-binding site[6], and illustrates the importance of performing these types of competition studies with different labeled ligands.

The consequence of the absence of binding of E_4 to human SHBG is that changes in the plasma levels of this protein will not influence the access of E_4 to its target tissues.

The ERα-dependent effect on SHBG production of the estrogens E_2, E_3, E_4 and EE was investigated using the HepG2 and Hep89 cell lines. Estetrol did not stimulate the production of SHBG in either cell lines, indicating that E_4 is not likely to influence the plasma levels of SHBG. This is important since the plasma distribution of the natural steroid ligands, such as E_2 and testosterone, or several synthetic progestins depends on the binding of these ligands to SHBG.

The other estrogens E_2, E_3 and EE all show a dose-dependent ERα-mediated increase in the production of SHBG. This increase in the SHBG production is the strongest for E_2, while addition of E_3 and EE resulted in a comparable increase in SHBG.

In addition, E_2 and EE also increase SHBG production in the HepG2 cell line, lacking the ERα. However, this is observed only after 72 h of incubation and at the highest concentrations.

The absence of a stimulatory effect of E_4 on the synthesis of SHBG suggests that E_4 may exhibit less interaction with liver function compared to other estrogens

Conflict of interest H.C.B. is CEO and shareholder of Pantarhei Bioscience; M.V. is employee and shareholder of Pantarhei Bioscience.

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